Biochemical Genetics (2019) 57:522–539
https://doi.org/10.1007/s10528-019-09904-6

ORIGINAL ARTICLE

Population Genetics of *Calotropis gigantea*, a Medicinal and Fiber Resource Plant, as Inferred from Microsatellite Marker Variation in two Native Countries

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Received: 3 September 2018 / Accepted: 4 January 2019 / Published online: 7 February 2019

Abstract

Calotropis gigantea is well known for its aesthetic, medicinal, pharmacological, fodder, fuel, and fiber production potential. Unfortunately, this plant species is still undomesticated, and the genetic information available for crop improvement is limited. For this study, we sampled 21 natural populations of *C. gigantea* from two key areas of its natural distribution range (Bangladesh and China) and genotyped 379 individuals using nine nuclear microsatellite markers. Population genetic diversity was higher in Bangladesh than that observed in Chinese populations. Overall, a moderate level of genetic diversity was found ($N_a = 3.73$, $H_E = 0.466$), with most of the genetic variation detected within populations (65.49%) and substantial genetic differentiation ($F_{ST} = 0.345$) between the study regions. We observed a significant correlation between genetic and geographic distances ($r = 0.287$, $P = 0.001$). The Bayesian clustering, UPGMA tree, and PCoA analyses yielded three distinct genetic pools, but the number of migrants per generation was high ($N_M = 0.52–2.78$) among them. Our analyses also revealed that some populations may have experienced recent demographic bottlenecks. Our study provides a baseline for exploitation of the genetic resources of *C. gigantea* in domestication and breeding programs as well as some insights into the germplasm conservation of this valuable plant.

Keywords *Calotropis gigantea* · Domestication · Genetic diversity · Gene flow · Microsatellites · Population bottleneck

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s10528-019-09904-6) contains supplementary material, which is available to authorized users.

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Introduction

Calotropis (Apocynaceae) is a small genus consisting of two species viz. *C. gigantea* (L.) W.T. Aiton and *C. procera* (Aiton) W.T. Aiton. *Calotropis gigantea* is native to the Indian sub-continent (Bangladesh, India, Pakistan, and Sri Lanka), China, Indonesia, Malaysia, Cambodia, Thailand, and the Philippines (Rahman and Wilcock 1991). The species has anti-influenzal, larvicidal, anti-bacterial, anti-fungal, and anti-cancerous bioactive properties (Parhira et al. 2014; Tariq et al. 2017). It is also used as a biofuel in arid and semi-arid regions and as a popular evergreen fodder (Abbas et al. 1992; Barbosa et al. 2014). The stem fiber is commercially used to make bowstrings, ropes, carpets, and fishing nets, while the seed fiber is used for making soft, stuffy mattresses, and pillows (Tanuj Kanchan and Alok Atreya 2016). The seed fiber also has the potential as reinforcing agent for manufacturing natural fiber composites, which is an attractive alternative to synthetic fiber composites in many applications (automobile parts, building materials, etc.) (Reddy and Yang 2009; Babu et al. 2014). Recently, *Calotropis* has achieved a great reputation as a new feedstock of fiber and drug production (Ashori and Bahreini 2009; Parhira et al. 2016; Muchugi et al. 2017). *Calotropis gigantea* is widely distributed, and the collection of samples for geographical characterization is difficult, expensive, and time-consuming. For this study, we analyzed samples from two key, geographically distant areas of its natural distribution range (Bangladesh and China), where the species is broadly used in traditional medicine (Motaleb et al. 2011; Parhira et al. 2016).

Understanding the genetic variation of *C. gigantea* in Bangladesh and China is interesting for several reasons. Genetic diversity of natural populations is an essential component of biodiversity that affects species survival, fitness, and evolution (Ellstrand and Elam 1993), in addition to represent a fundamental trait for crop improvement (Angelo et al. 2015; Tabkhkar et al. 2018). Besides, genetic information could inform conservation measures in particular areas of the species' natural distribution. For instance, Bangladesh is a small country with high human-mediated pressure (Worldometers 2018). *Calotropis gigantea* is extensively distributed in Bangladesh, and current threats such as habitat loss, fragmentation, and over-harvesting of the plant might impact the levels of genetic diversity in these areas. In China, the species is only found across the southern regions (e.g., Sichuan, Yunnan, Guangxi, Guangdong, and Hainan provinces; eFloras 2008), and fragmentation naturally occurs due to the presence of mountains, rivers, and islands. Gene flow is a very important factor in plant evolution that is affected by population dynamics, communities, ecosystem, anthropogenic pressures (urbanization, intensive agriculture, deforestation, habitat fragmentation, etc.), and natural barriers (hill and mountain ranges, river, shoreline, island, glacier, etc.) through hampering pollination and dispersal of seed (Hartl and Clark 1980).

Despite their ecological importance and commercial use, there are a limited number of studies addressing the distribution of genetic diversity in *Calotropis* species. Previous studies have analyzed the levels of intra-specific genetic

diversity using dominant RAPD markers in *C. gigantea* (Priya et al. 2015), and RAPD, AFLP, and ISSR markers in *C. procera* (Pandeya et al. 2007; El-Bakry et al. 2014; Angelo et al. 2015). Recently, Muriira et al. (2018) performed population genetic analyses of both *Calotropis* species using 20 EST-SSR markers. However, due to the widespread distribution of these species, genetic information for some areas of special interest is still lacking.

In this study, we genotyped 379 individuals of *C. gigantea* from 21 natural populations using nine nuclear microsatellite markers. Our goals were (a) to compare the level of genetic diversity between two geographically distant regions and between island and mainland populations, (b) to assess the global population structure of the study species, and (c) to estimate historical levels of gene flow between *C. gigantea* genetic pools. Our results were also interpreted in light of conservation issues and to guide germplasm collection efforts for this key plant.

Materials and Methods

Plant Materials and Sampling

Calotropis gigantea is a perennial evergreen shrub or small tree attaining a height of 3 to 4 m, and with potential to occupy disturbed areas such as roadsides, railway lines, fallow lands, riverbanks, and coastal sides. It is a diploid ($2n = 22$), outcrossing weed and its reproductive success mostly depends on pollination by Hymenopteran insects (Ali and Ali 1989). Its widespread distribution can be explained by its ample tolerance to both drought and salt stress, its ability for sexual (via seeds) and clonal reproduction, and its morphological adaptations for wind dispersal of seeds (Francis 2003; Ramadan et al. 2014).

For this study, we sampled 21 *C. gigantea* populations. Ten populations comprised all of the main regions occupied by the species in China, and 11 additional populations represented the main phytogeographical regions of Bangladesh, with a total sample size of 379 individuals. Three of 21 *C. gigantea* populations (54 individuals) sampled from Hainan Island, China and rest of the populations collected from mainland counterparts. Sampled individuals were separated by a distance of 20 to 50 m, depending on the size of the population (Supplemental file 1—Table S1 and Fig. 1). A voucher specimen was taken for each population and preserved at the Wuhan Botanical Garden Herbarium, Chinese Academy of Sciences.

DNA Extraction and SSR Genotyping

Total genomic DNA was extracted from about 20 mg of leaf tissue per individual, using modified CTAB procedure (Doyle and Doyle 1987). In order to analyze levels of genetic diversity from the individuals sampled in natural populations, we initially screened 23 microsatellite loci (Muriira et al. 2015) that were selected to represent dinucleotide, tri-nucleotide, and tetra-nucleotide motifs and the PCR

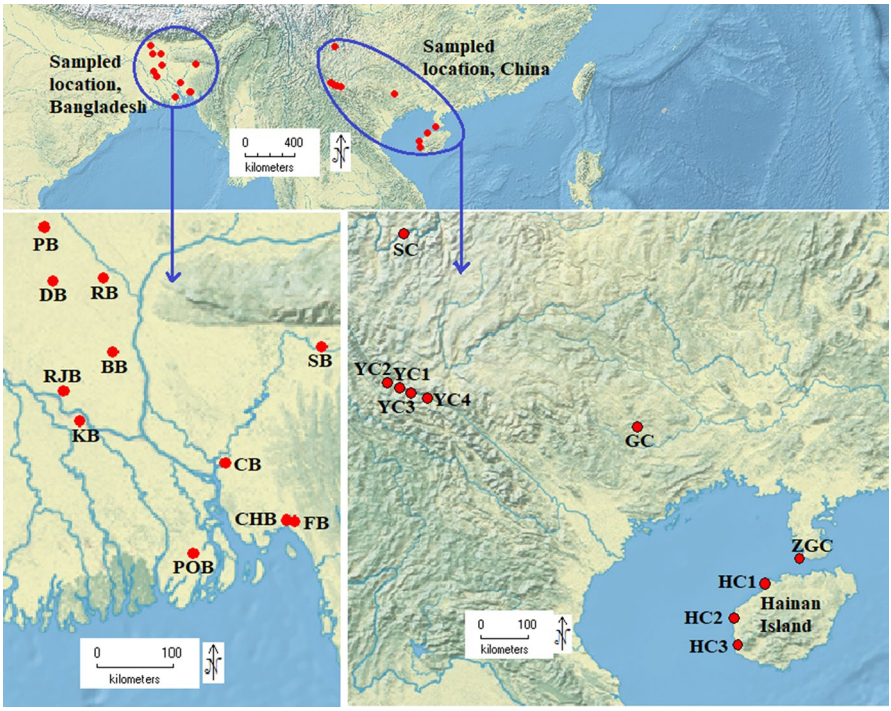

Fig. 1 Geographic location of 21 *C. gigantea* populations sampled from Bangladesh and China. Population codes are identified in Supplemental file 1—Table S1

results were checked in a 2.0% agarose gel. Seven microsatellites did not amplify (c6776, c19693, c12045, c12090, c13112, c19025, and c11368). The remaining loci were used to genotype 24 individuals from three populations (one from Bangladesh, one from mainland China, and other from Hainan Island with eight individuals). Among these 16 SSRs, only nine were polymorphic and interpretable, and were therefore selected for genotyping the total number of individuals. The features of these nine microsatellite loci are presented in Supplemental file 1—Table S2, and Figs. S1 and S2. Forward primers were labeled with a fluorochrome (FAM) dye on the 5′ end. The PCR was performed in a total volume of 25 µL that contained 2 µL (approx. 50 ng) of genomic DNA, 2.5 µL 10× Taq buffer (plus Mg^{2+}), 0.50 mM of each dNTP, 0.50 µM of each primer, 0.2 U of Taq polymerase (Beijing TransGen Biotech Co., Ltd., China), and deionized water. The amplification was performed using a T100™ Thermal Cycler (Bio-Rad, CA, USA) with the following steps; denaturation for 5 min at 95 °C, followed by 30 cycles of 30 s at 95 °C, 30 s at 53–55 °C, 30 s at 72 °C, with a final extension step for 10 min at 72 °C. The quality of PCR products was checked in a 2.0% agarose gel and separated by an ABI 3730 XL automated sequencer (Wuhan GeneCreate

Biological Engineering Co. Ltd., Wuhan, China). Allele scoring was performed using GeneMarker 2.6.3 (SoftGenetics).

Data Analyses

Microsatellites Data Testing

Micro-Checker v0.2.2.3 was used to detect potential artifacts associated with stuttering or large allele dropout, and also to estimate the null-allele frequency (NAF) at each locus (van Oosterhout et al. 2004). Due to the observation of low NAF in four loci (see "Results" section), we used FreeNA (Chapuis and Estoup 2007) to produce corrected data for the rest of the genetic analyses (see below). Genetic parameters were calculated from the original dataset (Supplemental file 2—Data S1).

Genetic Diversity Estimates

Genetic diversity parameters such as average number of observed alleles (N_a), average number of effective alleles (N_e), Shannon's information index (I), observed heterozygosity (H_O), expected heterozygosity (H_E), and number of private alleles were analyzed using GenAlEx 6.5 (Peakall and Smouse 2012). Cervus 3.0 was used to calculate the polymorphic information content (PIC) (Kalinowski et al. 2007), and 'Genepop on the web' software (Rousset 2008) was used to test for Hardy–Weinberg equilibrium (HWE) conditions with Bonferroni corrections for each locus. Statistical comparisons of genetic diversity between Bangladesh versus China and mainland versus island were calculated using FSTAT 2.9.3.2 (Goudet 2001), using allelic richness (A_R), observed heterozygosity (H_O), gene diversity (H_S), inbreeding coefficient (F_{IS}), and genetic differentiation (F_{ST}) values. Inbreeding coefficients without null-allele correction (F_{IS}) and with null-allele correction ($F_{IS}^{\{IIM\}}$) were calculated using FSTAT and INEST 2.2 (Chybicki and Burczyk 2009), respectively. The latter is a robust approach for datasets affected by large frequencies of null alleles. For this analysis, we followed a Bayesian procedure individual inbreeding model (IIM) with 500,000 cycles, n-th update (thinning) 1000 to avoid strong autocorrelation for getting a good approximation of the posterior distributions, and burn-in 50,000 to estimate deviance information criterion (DIC) values for selecting 'nfb' full model (n, f, and b are represented null alleles, inbreeding coefficients, and genotyping failures, respectively). Mean F_{IS} and $F_{IS}^{\{IIM\}}$ values were compared with a paired samples t-test using the SPSS software version 23.0 (IBM Corp. Released 2015).

Population Structure

We used FreeNA for estimating global and pairwise population differentiation using both ENA (excluding null allele) correction ($F_{ST}^{\{ENA\}}$) and without ENA correction (F_{ST}) (Chapuis and Estoup 2007). Analysis of molecular variance (AMOVA) was performed with ARLEQUIN ver. 3.1 (Excoffier et al. 2005). Mantel's test for IBD was examined using matrix correlation between null corrected linearized F_{ST}

($F_{ST}^{\{ENA\}}/(1 - F_{ST}^{\{ENA\}})$) versus log geographic distance (km) with 999 permutations by GenAlEx 6.5. To test for bottleneck effects across *C. gigantea* populations, we used BOTTLENECK 1.2.02 (Piry et al. 1999). The analyses were run assuming a stepwise mutation model (SMM) and a two-phase model (TPM-70% SMM and 30% IAM (infinite allele model)). Statistical significance was assessed with the sign test, Wilcoxon signed-rank test, and the allele frequency distribution mode shift (Cornuet and Luikart 1996; Luikart and Cornuet 1998). A PCoA was performed in GenAlEx 6.5 using pairwise $F_{ST}^{\{ENA\}}$ genetic matrixes. Cavalli-Sforza and Edwards genetic distance (Cavalli-Sforza and Edwards 1967) for each pair of populations with INA (i.e., including null allele) correction were calculated by FreeNA to construct a UPGMA dendrogram using MEGA7 (Kumar et al. 2016). A Bayesian approach was used to estimate the number of genetic clusters or pools and individual-based differentiation with the program STRUCTURE 2.3.4 (Pritchard et al. 2000). The program was run following the admixture (allele frequencies correlated) and non-admixture (allele frequencies independent) model, and without prior information about the populations with 500,000 MCMC (Markov Chain Monte Carlo) repetitions, with a burn-in period of 50,000 and ten replicates; preliminary simulations K value ranging from 2 to 21. We run non-admixture model due to the presence of inbreeding in *C. gigantea* and populations were deviated from HWE expectation (Szczecińska et al. 2016). Based on ΔK statistics (Evanno et al. 2005), the optimum value of population clusters (K) was calculated using Structure Harvester (Earl and vonHoldt 2012).

Gene Flow Estimation

We used Migrate-n Ver. 3.6.11 (Beerli 2012) for determining historical gene flow as number of migrants per generation (N_M) considering the population clusters defined by Structure. We applied the coalescent and Bayesian inference approach (Beerli 2006) with thermodynamic integration, Markov Chain Monte Carlo (MCMC) simulation, Brownian approximation method, and a constant mutation rate for all loci. The initial parameters for θ and M values were generated from the F_{ST} calculation (Beerli and Felsenstein 1999). The analysis was carried out in a single Markov long chain with 1,000,000 discarded trees per chain (burn-in), 500,000 visited (sampled) parameter values, and 5000 recorded steps. MIGRATE-n yielded the mutation-scaled effective population size Θ ($4N_e\mu$) and the mutation-scaled migration rate M (m/μ), where, N_e, μ, and m are the effective population size, mutation rate per locus in a generation, and immigration rate among populations in a generation, respectively (Beerli and Palczewski 2010). The N_M was calculated as the multiplication of Θ and M, divided by 4 ($N_{Mj \to i} = [\Theta i \times M_{j \to i}]/4$).

Results

Microsatellite Variation

Genetic variation as obtained from the amplification of nine microsatellite loci is given in Supplemental file 1—Table S3. A total of 151 alleles were detected in 379 individuals of *C. gigantea*. The highest PIC was found at locus Cag22 (0.867) and the lowest at locus Cag16 (0.397), with a mean across loci of 0.695. Estimates of population differentiation (F_{ST}) with and without ENA correction across all loci were 0.331 and 0.318, respectively. Micro-checker analyses

Table 1 Genetic diversity of each sampled population over nine microsatellite loci for *C. gigantea*

Populations	N_a	N_e	H_O	H_E	I	Private alleles	Inbreeding coefficient	
							F_{IS}	$F_{IS}^{\{IIM\}}$
DB	4.56	2.93	0.470	0.634	1.186	6	0.283	0.208
PB	4.22	2.54	0.471	0.528	0.995	3	0.134	0.114
RB	3.00	2.18	0.374	0.457	0.803	0	0.202	0.041
BB	4.56	2.81	0.522	0.544	1.060	3	0.065	0.053
CB	6.00	3.36	0.403	0.682	1.403	9	0.432	0.347
SB	4.00	2.20	0.313	0.457	0.889	4	0.349	0.118
CHB	3.56	2.45	0.319	0.534	0.967	1	0.441	0.268
FB	6.00	3.25	0.480	0.650	1.321	9	0.283	0.218
KB	4.22	2.38	0.370	0.506	0.967	2	0.292	0.282
POB	4.44	2.18	0.381	0.486	0.952	3	0.241	0.147
RJB	5.22	2.66	0.375	0.561	1.133	5	0.355	0.251
SC	4.11	2.68	0.339	0.480	0.935	2	0.319	0.360
YC1	1.89	1.36	0.099	0.189	0.324	0	0.505	0.264
YC2	1.89	1.30	0.114	0.150	0.273	1	0.267	0.151
YC3	2.67	1.61	0.275	0.299	0.531	1	0.109	0.028
YC4	2.78	1.84	0.282	0.391	0.658	0	0.310	0.144
HC1	2.44	1.73	0.313	0.362	0.607	0	0.172	0.261
HC2	2.56	1.83	0.162	0.378	0.633	0	0.590	0.581
HC3	3.11	2.14	0.336	0.465	0.800	1	0.302	0.253
GC	4.00	2.77	0.496	0.584	1.085	1	0.201	0.076
ZGC	3.00	2.22	0.331	0.453	0.786	3	0.302	0.059
Overall mean	3.73	2.31	0.344	0.466	0.872	2.57	0.293	0.201
Mean (Bangladesh)	4.53	2.63	0.407	0.549	1.061	4.09	0.280	0.186
Mean (China)	2.85	1.95	0.275	0.375	0.663	0.90	0.308	0.218
Mean (Mainland)	3.89	2.37	0.356	0.477	0.904	2.944	0.283	0.174
Mean (Island)	2.70	1.90	0.270	0.402	0.680	0.333	0.355	0.365

N_a average number of observed alleles, N_e average number of effective alleles, H_O observed heterozygosity, H_E expected heterozygosity, I Shannon's information index, F_{IS} Inbreeding coefficient without null correction, $F_{IS}^{\{IIM\}}$ inbreeding coefficient followed null corrected individual inbreeding model

did not detect stuttering effects or large allele dropouts. All tested loci were significantly deviated from HWE expectations, thus indicating a heterozygote deficit condition. Five loci did not show significant NAF values (ranges 0.02 to 0.05 ≤ 0.05), whereas four loci displayed low-frequency null alleles (0.13 to 0.18). Overall, NAF was low (mean = 9%) across 21 populations (Supplemental file 1—Table S4).

Population Genetic Diversity

Genetic diversity estimates of the study *C. gigantea* populations are shown in Table 1. The N_a ranged from 1.89 (YC1 and YC2) to 6.00 (CB and FB) with a mean across populations of 3.73, whereas the N_e varied from 1.30 (YC2) to 3.36 (CB) with a mean across populations of 2.31. The H_O was diverse from 0.099 (YC1) to 0.522 (BB) with a mean variation of 0.344. The mean H_E was 0.466 which ranged from 0.150 (YC2) to 0.682 (CB). The average value of Shannon's information index and number of private alleles were 0.872 and 2.57, respectively. The inbreeding coefficient, F_{IS} ranged from 0.065 to 0.590 with an average 0.293 that significantly varied with $F_{IS}^{\{IIM\}}$ (ranged 0.028 to 0.581 with a mean 0.201) (significant test data not given).

Table 2 Analysis of molecular variance (AMOVA) of genetic diversity of *C. gigantea* populations

Sources of variation	df	Sum of squares	Variance components	Percentage of variation	F_{ST}
(a) Total variation					
Among all populations	20	821.862	1.08330 Va	34.51	0.345***
Within population	737	1515.312	2.05605 Vb	65.49	
(b) Bangladesh versus China					
Between countries	1	126.116	0.23786 Va	7.31	0.368***
Among populations within countries	19	695.746	0.95998 Vb	29.50	
Within populations	737	1515.312	2.05605 Vc	63.19	
(c) Mainland versus Island					
Between regions	1	160.085	0.67147 Va	18.46	0.435***
Among populations within regions	19	661.777	0.91073 Vb	25.03	
Within populations	737	1515.312	2.05605 Vc	56.51	
(d) Three groups as in STRUCTURE					
Among groups	2	365.688	0.73210 Va	21.32	0.401***
Among populations within group	18	456.174	0.64598 Vb	18.81	
Within populations	737	1515.312	2.05605 Vc	59.87	

df degree of freedom

***$P < 0.001$

Population Genetic Differentiation and Structure

The pairwise $F_{ST}^{\{ENA\}}$ estimates of population differentiation ranged from 0.059 (CB and FB) to 0.698 (YC2 and HC1), with a global $F_{ST}^{\{ENA\}} = 0.323$ (Supplemental file 1—Table S5). Hierarchical AMOVA (Table 2) showed that most of the genetic variation was found within populations (65.49%), whereas the among-population component explained a smaller fraction (34.51%). When genetic groups were considered, variation between groups explained 21.32% of the total variation. In PCoA, broadly, two main groups observed (Bangladeshi and Chinese populations formed distinctive group) with a high variation (48.90%), suggested that a discrete genetic structure occurred in *C. gigantea* populations. The first axis with 22.85% variation separated the Chinese populations into two groups, while the second axis with 15.26% variation isolated the Bangladeshi populations of *C. gigantea* (Supplemental file 1—Fig. S3). The UPGMA tree based on Cavalli-Sforza and Edwards's genetic distance with INA correction produced a clear clustering of the populations,

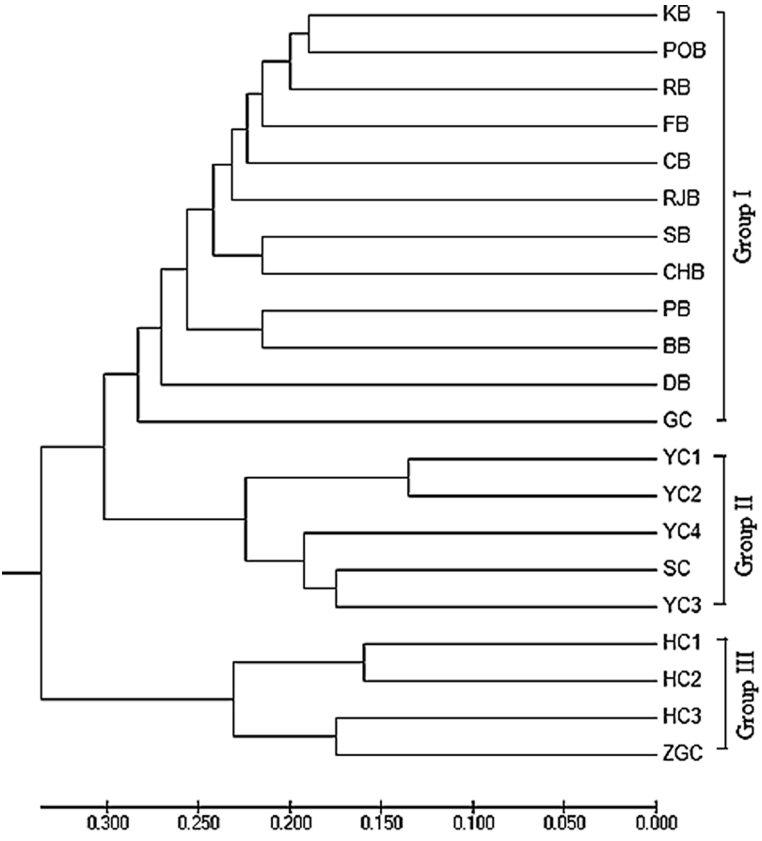

Fig. 2 UPGMA dendrogram based on Cavalli-Sforza and Edwards genetic distance with INA (i.e., including null allele) correction estimated among 21 populations of *C. gigantea*

with clusters that roughly corresponded with Structure results (Fig. 2). The linear correlation between linearized genetic differentiations versus log geographic distances was significant ($r = 0.287$, $P = 0.001$) (Supplemental file 1—Fig. S4). Recent demographic bottlenecks were observed in four populations (CB, POB, RJB, and ZGC) under SMM and TPM models, and in two populations (RB and HC1) according to normal L-shaped distribution (Supplemental file 1—Table S6). The STRUCTURE analysis of *C. gigantea* was performed for population stratification followed by Bayesian clustering of admixture and non-admixture model, which yielded three genetic pools among 21 populations ($K = 3$) in both cases (Supplemental file 1—Fig. S5a and S5b). Both of the model yielded similar genetic clustering of populations. This clustering placed 12 sample sites (DB, PB, RB, BB, CB, SB, CHB, FB, KB, POB, RJB, and GC) in Group I, 5 sample sites (YC1, YC2, YC3, YC4, and SC) in Group II, and 4 sample sites (HC1, HC2, HC3, and ZGC) in Group III (Fig. 3a, b).

Historical Gene Flow

The pattern of historical gene flow among the three genetic groups of populations outlined by STRUCTURE is presented in Supplemental file 1—Table S7 and Fig. S6. The Θ values (+ 97.5% confidence intervals) for Group I, II, and III were 0.098 (0.100), 0.071 (0.013), and 0.017 (0.021), respectively. The highest number of migrants (2.78) was found from Group I to Group II, whereas the lowest number of migrants (0.52) was found from Group II to Group III.

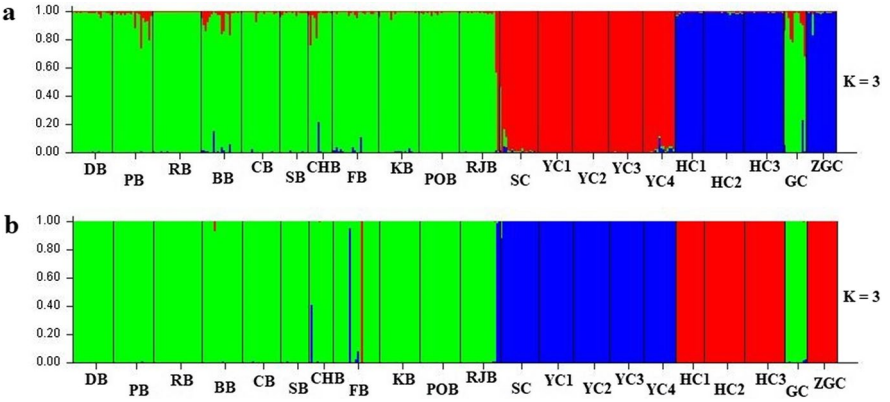

Fig. 3 STRUCTURE analysis of nSSR data generated for 21 populations of *C. gigantea.* Results of genetic clustering are shown for admixture (**a**) and non-admixture (**b**) models

Discussion

Informativeness and Justification of Microsatellites

The deviation of nSSR from HWE might be influenced by inbreeding, Wahlund effects, or the presence of null alleles. Based on PIC (Botstein et al. 1980) criterion, the nSSR markers exhibited a high level of polymorphism (mean PIC = 0.695) that was supported by Muriira et al. (2018). Like most of the taxa (Dakin and Avise 2004), *C. gigantea* experienced low level of null alleles, which attributed very less differences in genetic differentiation across all loci and gave similar Bayesian clustering of populations for admixture and non-admixture models, indicated null alleles have very little affect in our analyses (Hauser et al. 2006; Carlsson 2008). We observed 16 of 21 populations of *C. gigantea* contained private alleles (Table 1) explaining that the nSSR are useful for evolutionary analyses of this species, which is consistent with *Petunia* (Turchetto et al. 2015). Overall, the nSSR markers exhibited satisfactory polymorphism across the loci for performing population genetics estimations.

Genetic Diversity of *C. gigantea* Across the Study Areas

Calotropis gigantea showed significantly higher estimates of genetic diversity (A_R, H_O, and H_S) in Bangladesh than those found among Chinese populations (Supplemental file 1—Table S8). The highest estimates of genetic diversity were found in Comilla, Bangladesh (H_E = 0.682 and N_e = 3.36) and the lowest in Yuxi-2, Yunnan, China (H_E = 0.150 and N_e = 1.30). Bangladesh provides favorable climates and suitable habitats for *C. gigantea* since about 80% of the territory is composed of lowland plains with very few physical barriers (i.e., mountain areas are restricted to the Eastern and South-eastern regions of the country). Thus, availability of suitable habitats and potential for wind seed dispersal across large areas likely promotes accumulation of genetic diversity and population connectivity (García-Verdugo et al. 2018). The lower genetic diversity of *C. gigantea* in Southern China is probably influenced by barriers such as mountains, and rivers, which may be responsible for the lower rates of gene flow and higher inbreeding detected among these populations.

In our study, we compared the genetic diversity of all mainland (Bangladesh plus China) versus island populations, and also between mainland and island Chinese populations. In both cases, mainland populations showed slightly higher genetic diversity than island populations, but differences were not statistically significant (Supplemental file 1—Table S8). These results are consistent with general patterns of genetic diversity. On one hand, outcrossing, wind-dispersal species with wide distribution ranges typically show high levels of genetic diversity (Nybom 2004). In addition, island genetic diversity strongly depends on population size rather than on the island condition per se (Maki and Morita 1998; García-Verdugo et al. 2015). These attributes together (breeding system, dispersal ability, and population

size) may explain why levels of genetic variation between island and mainland *C. gigantea* populations are similar.

However, *C. gigantea* showed a moderate level of mean genetic diversity across populations (N_a = 3.73 and H_E = 0.466), which is consistent with the findings of Muriira et al. (2018). Although Su et al. (2017) also found moderate levels of diversity in the insect pollinated, small tree, *Tamarix taklamakanensis*, genetic diversity in *C. gigantea* is lower than that described for other outcrossing species in the Apocynaceae family (i.e., Kabat et al. 2010; Nakahama et al. 2012; Yamashiro et al. 2016).

The inbreeding coefficient of *C. gigantea* may explain such moderate levels of genetic diversity. Homozygosity excess might result from sampling biases, Wahlund effects, population bottlenecks, or the presence of null alleles. INEST gave slightly lower inbreeding coefficient with a mean $F_{IS}^{\{IIM\}}$ = 0.201 (ranging from 0.028 to 0.581), than that obtained with FSTAT (F_{IS} varied from 0.065 to 0.590, mean 0.293) indicated that null allele has limited influence on the inbreeding coefficient (Maebe et al. 2013; Wei et al. 2013). Our results suggested that inbreeding is a common phenomenon among *C. gigantea* wild populations. Muriira et al. (2018) also obtained significant inbreeding coefficients (0.234) in *C. gigantea*. *Calotropis gigantea* has larger floral displays, and produces large amounts of inflorescences within a single individual. This likely increases pollinator (*Xylocopa* and *Apis* spp.) movement within the same individual (Ali and Ali 1989), which facilitates self-fertilization (Menge et al. 2017).

Hierarchical Distribution of Genetic Variation in *C. gigantea*

Calotropis gigantea showed higher within population genetic variation (65.49%) than among populations (34.51%), which could be associated with the species life history traits such as woody shrub or small tree, perennial weed, outcrosser, and anemochory (Hamrick et al. 1992). These results parallel those obtained for some other Apocynaceae species, such as *Aspidosperma polyneuron* (Torezan et al. 2005), *Asclepias meadii*, *Asclepias tuberosa Asclepias incarnata*, and *Asclepias viridis* (Comer 2009), *Mandevilla velutina* (Bertoni et al. 2010), and *Asclepias tuberosa* (Boylan et al. 2009). The AMOVA output showed a strong genetic structure which was supported by the high level of genetic differentiation (F_{ST} = 0.345) that was slightly higher with ENA corrected differentiation ($F_{ST}^{\{ENA\}}$ = 0.323). The higher genetic differentiation (F_{ST} > 0.25) of *Solanum rostratum* and *Lactuca watsoniana* weed species also supported our findings (Zhao et al. 2013; Dias et al. 2016). Muriira et al. (2018) explained the genetic differentiation of *C. gigantea* (F_{ST} = 0.570) which was higher than our output. However, high genetic differentiation in *C. gigantea* might be the cause of restricted gene flow due to the presence of natural barriers that lead to hinder the insect pollinators' movement to far distance. The genetic makeup of a species is greatly influenced by its spatial (geographic isolation) and temporal diversity (Smith 1999). In Mantel test, we perceived a significant correlation between genetic and geographic distances (r=0.287, P =0.001) that indicated sampled populations separated by greater distances were structured

both spatially and genetically; and IBD played a role in genetic differentiation of *C. gigantea*. The IBD indicates that we need to select genetically diverse genotypes of *C. gigantea* during future domestication and breeding program. Muriira et al. (2018) reported insignificant correlation for IBD in *C. gigantea* that might be the effect of limited number of populations. However, our results in consistent with the IBD of *Populus simonii* ($r = 0.262$, $P < 0.05$), which is a widely distributed, outcrossing, and wind-dispersal tree (Wei et al. 2013). The demographic bottleneck is found in a population with sudden decreases of population size due to habitat loss, alteration, fragmentation, and natural calamities. The bottleneck is responsible for reducing genetic diversity by decreasing heterozygosity due to random drift and inbreeding of the populations (Nei et al. 1975). We evaluated that there were four populations (RB, CB, POB, and RJB) from Bangladesh and two populations (HC1 and ZGC) from China showed recent past bottleneck. Among 11 populations from Bangladesh, 4 populations showed bottleneck that focused *C. gigantea* is facing more anthropogenic and natural pressures in Bangladesh compared to China. Generally, bottleneck is common in island populations rather than mainland populations due to founder effect. Our results revealed a new recent impact on mainland populations of *C. gigantea*, which are widely distributed and outcrossing, but experienced with bottlenecks like other common plant species such as *Ficus pumila* and *Ardisia crenata* var. *bicolor* (Zhao et al. 2006; Chen et al. 2008).

The Bayesian analysis displayed a strong genetic structure among 21 populations of *C. gigantea* by clustering into three distinct genetic pools. The populations from Bangladesh and China made separate groups, except GC (sampled from Guangxi, China) which bundled with the other populations of Bangladesh (Group I). The rest of the populations collected from China isolated into two groups. The populations from Yunnan and Sichuan provinces clustered in Group II. The island populations shaped Group III with Guangdong indicated that *C. gigantea* in Hainan Island might be probably originated from Guangdong. The separate grouping of the island sites for *C. gigantea* rhyme with genetic grouping of *Saccharum spontaneum* species in China (Fan et al. 2013). The UPGMA and PCoA analysis also provided similar genetic clustering of the populations. The knowledge about the genetic structure of *C. gigantea* natural population is essential for their domestication, improvement, and germplasm conservation (Moura et al. 2011).

High Number of Migrants Per Generation Among Genetic Pools

The N_M between most of the groups were high (1.21 to 2.78 > 1.00) except from Group II to III and from Group I to III (0.52 and 0.91, respectively) directed a stable gene flow among populations. This gene flow pattern was higher than other Apocynaceae plant species like *Hancornia speciosa* (1.18), *Vincetoxicum atratum* (1.463), and *Gymnema sylvestre* (0.21 to 0.33) (Jimenez et al. 2015; Yamashiro et al. 2016; Rathore et al. 2016). The outcrossing system and wind dispersal of seed facilitate higher historical gene flow in *C. gigantea*. Some researchers also mentioned that $N_M > 1$ or 1 to 10 migrants per generation are effective for restoration, reducing genetic

drift, and act against natural selection and increase the population fitness for survival (Slatkin 1985; Blanquart et al. 2011). High gene flow is an indication of the adaptability of *C. gigantea* against diverse environments and its effective colonization.

Conclusion

A moderate genetic diversity with substantial population structure and moderate levels of gene flow were observed among *C. gigantea* populations. Fragmentation is not threatening only for rare plant species but also for common habitat specialists (Lienert and Fischer 2003), and therefore, we suggested to germplasm collection and conservation of the genetic resources of *C. gigantea* in such small countries like Bangladesh, where habitat fragmentation occurs rapidly due to high population pressure.

Acknowledgements We acknowledged Professor Dr. Md. Abu Hasan, Hajee Mohammad Danesh Science and Technology University, Bangladesh, and Xu Zhun for helping during sample collection; Shu-Ying Zhao for assistance in data analyses; and John Mulinge Nzei and Mwanzia Virginia M. for language editing of the manuscript. This project financially supported by National Natural Science Foundation of China (31670226) and CAS-TWAS President's PhD Fellowship Program, University of Chinese Academy of Sciences, China.

Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

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