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The phylogeographic history of the self-pollinated herb *Tacca chantrieri* (Dioscoreaceae) in the tropics of mainland Southeast Asia

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ABSTRACT

The geological and climatic oscillations influenced the geographic distribution and demography of most present-day species, but few studies have investigated evolutionary history of species adapted to the tropical regions of Southeast Asia. Here, using sequence datasets obtained from three chloroplast DNA fragments (*trn*H-*psb*A, *trn*S-*trn*G, and *trn*L-F) from 320 individuals belonging to 24 natural populations, we investigated the phylogeographical history of *Tacca chantrieri*, which inhabits Southeast Asian tropical forests. Although relatively high level of differentiation among the populations were observed, mismatch distribution and neutrality tests showed no evidence of recent demographic population expansion. Phylogenetic inference exhibited two identified population groups showing a disjunctive distribution of dominant haplotypes. The split in cpDNA was largely consistent with the Tanaka line and Red River geographically. Molecular clock estimations revealed that the two lineages diverged during Pleistocene approximately 1.16 Ma. Therefore, the disjunct distribution of *T.chantrieri* could be explained by both the vicariance caused by Red River as well as ecological barriers caused by the different monsoon climates (Southwest monsoon *vs*. Southeast monsoon) that developed during the Pleistocene. The Tanaka line can be considered as a climatically driven barrier that influenced present-day plant dispersal.

1. Introduction

The current geographical distribution of living organisms were influenced by both present and past ecological or historical factors (Cheng et al., 2005) such as climatic oscillations of the Quaternary (Avise, 2000; Hewitt, 2000, 2004) and various geographical barriers (Demesure et al., 1996; Taberlet et al., 1998; Hiramatsu et al., 2001). Mainland Southeast Asia, is one of the 25 global biodiversity "hotspots" (Myers et al., 2000), which contains several important biogeographical boundaries and harbors high levels of biodiversity. Yunnan is a special region of complex geological history in southwestern China because it located at a sutural zone between Gondwana and Laurasia (Audley-Charles, 1987; Jin, 2002; Metcalfe, 2006). Location in this transitional zone between tropical south-east Asia and temperate east Asia allows for the high biodiversity in the region

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(Takhtajian, 1978; Wu and Wu, 1996). The origin and evolution of the Yunnan flora is considered to be largely influenced by several factors including the uplift of the Himalayas, the formation of the east Asian monsoon climate and the extrusion of the Indochina block into tropical Southeast Asia since the later Tertiary period (Zhu, 2012, 2013).

Many studies focused on the flora of Yunnan and its relationship to biogeography and climate of the neighboring areas. Among these, three main studies stand out. First, Li et al. (1999) pointed out that the formation of the tremendous physical and climatic changes were associated with the vertical displacement of Shan-Malay Plate. The complex movements of the tectonic plates resulted in many important biogeographical boundaries in Yunnan and neighboring areas, which could facilitate the vicariance and speciation of plants in this region. For example, the Tanaka line (TKL) (Fig. 1; Tanaka, 1954). Indeed, strong genetic differentiation across southern and northern parts of the TKL has been reported previously (Qiu et al., 2009; Guan et al., 2010). Second, Mitsui et al. (2008) ascribed the evolutionary history of Yunnan plants to both geological diversity as well as the climate in the distribution area of these species. The development of the Himalayas drove a large amount of rainfall by forming a monsoon system in East Asia (Sun and Wang, 2005). Southwest China is the geographical meeting place for the southeast (SE) monsoon and southwest (SW) monsoon providing climatic diversity to this area (Gao et al., 1962).

Third main idea that explores the Yunnan flora and its relationship to the biogeography and climate of the neighboring areas is the Red River hypothesis (Clark et al., 2004). The Red River, also known as the Hồng Hà or Sông Cái in Vietnamese and the Yuan River in Chinese, is a river that flows from Yunnan in southwest China through northern Vietnam to the Gulf of Tonkin (Fig. 1). Clark et al. (2004) proposed that the Jinshaijang (Upper Yangtze) and its tributaries (Yalongjiang, Daduhe, Jialingjiang), as well as the Salween (Nujiang), Mekong (Lancangjiang), and Brahmaputra (Yarlung Tsangpo), were all once tributaries to the paleo-Red River. Moreover, the past changes in paleo-drainage systems of ancient Red River could influence the phylogeographic structure of Yunnan flora (e.g. *Terminalia franchetii*; Zhang et al., 2011b).

Plant phylogeographical studies are capable of examining the distribution of genealogical lineages and assessing the relative importance of different historical events, such as range expansion/contraction, fragmentation, and migration from refugia (Taberlet et al., 1998; Hewitt, 2000; Morris et al., 2010). Qiu et al. (2011) reviewed the current literature about plant molecular phylogeography in China and adjacent regions and documented the population histories of temperate plant species. However, studies on phylogeography have most often been directed at woody plants (Wang and Ge, 2006; Yu and Nason., 2013; Zhao et al., 2013). Herbaceous plants may have been more sensitive to quaternary (historical) climatic oscillations than woody plants because of their short life cycles, and would therefore be ideal candidates to illustrate the evolutionary history of the flora (Meng and Zhang, 2011). However, very few studies have investigated the phylogeographic patterns of herbaceous plants in the tropical rain forests of Southeast Asia.

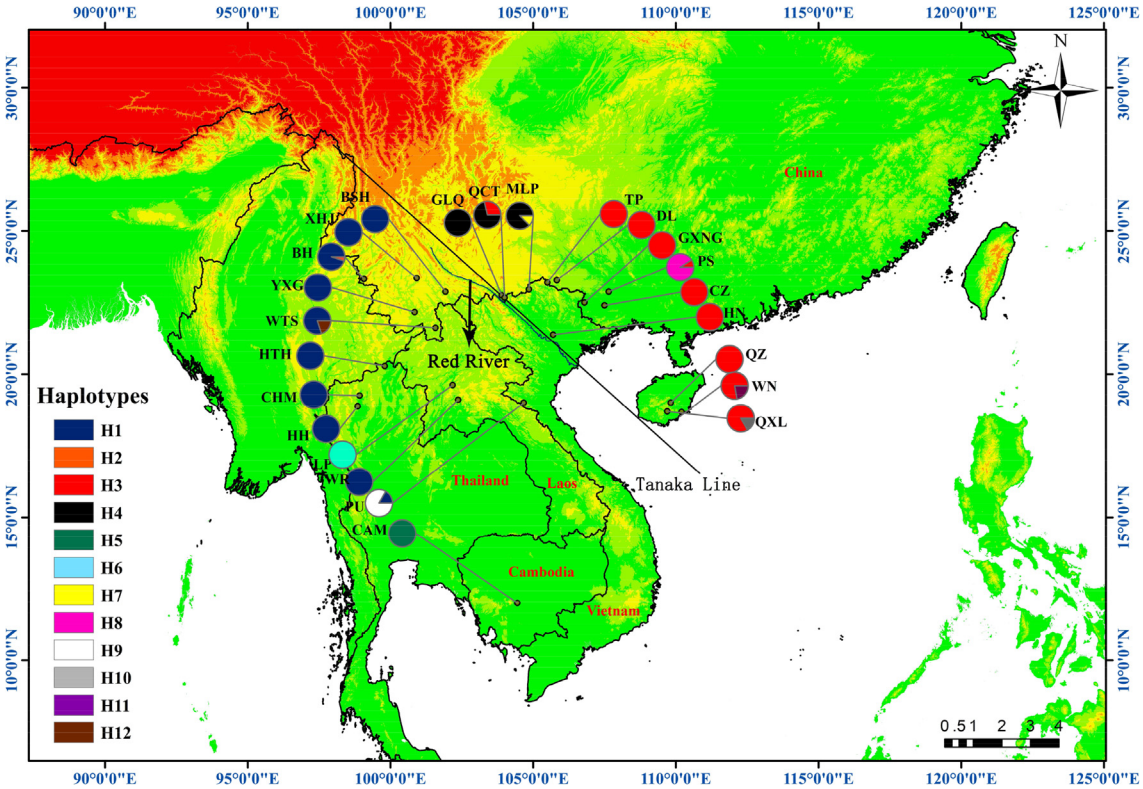

Fig. 1. Distribution of cpDNA haplotypes detected in 24 populations of *T. chantrieri*. Pie charts show the frequencies of haplotypes in each population.

Table 1
Population descriptions, estimates of haplotypes (H_d), and nucleotide diversity (π) of all sampled populations of *T. chantrieri*.

Pop. code	Sample size (N)	Locality	Region	Latitude (N), longitude (E)	Altitude (m)	Haplotypes (number of individuals)	H_d	π
1. GLQ	14	Gulinqing, Maguan, Yunnan, China	E	22°45′N, 103°58′E	700–1200	H4 (14)	0.00000 ± 0.00000	0.00000 ± 0.00000
2. MLP	16	Malipo, Yunnan, China	E	22°58′N, 104°51′E	800	H4 (14), H7 (2)	0.233 ± 0.126	0.00037 ± 0.00020
3. QCT	14	Qincaitang, Hekou, Yunnan, China	E	22°40′N, 104°01′E	680–780	H3 (4), H4 (10)	0.440 ± 0.112	0.00047 ± 0.00012
4. CZ	18	Chongzuo, Yunnan, China	E	22°24′N, 107°30′E	100	H3 (18)	0.00000 ± 0.00000	0.00000 ± 0.00000
5. DL	12	Delong, Yunnan, China	E	23°17′N, 105°50′E	600	H3 (12)	0.00000 ± 0.00000	0.00000 ± 0.00000
6. GXNG	10	Nonggang, Guangxi, China	E	22°32′N, 106°49′E	270	H3 (10)	0.00000 ± 0.00000	0.00000 ± 0.00000
7. HN	10	Hanoi, Vietnam	E	21°23′N, 105°42′E	10	H3 (10)	0.00000 ± 0.00000	0.00000 ± 0.00000
8. PS	11	Pingshan, Guangxi, China	E	22°53′N, 107°39′E	200	H3 (1), H8 (10)	0.182 ± 0.144	0.00010 ± 0.00008
9. QXL	12	Qixianling, Hainan, China	E	18°42′N, 109°42′E	900	H3 (10), H10 (2)	0.303 ± 0.147	0.00016 ± 0.00008
10. QZ	16	Qiongzhong, Guangxi, China	E	19°00′N, 109°49′E	350	H3 (16)	0.00000 ± 0.00000	0.00000 ± 0.00000
11. TP	12	Tianpeng, Yunnan, China	E	23°12′N, 105°32′E	900	H3 (12)	0.00000 ± 0.00000	0.00000 ± 0.00000
12. WN	16	Wanning, Guangxi, China	E	18°41′N, 110°12′E	80	H3 (12), H11(4)	0.400 ± 0.114	0.00021 ± 0.00006
13. BH	19	Banhong, Yunnan, China	W	23°12′N, 99°04′E	900	H1 (18), H2 (1)	0.10500 ± 0.09200	0.00006 ± 0.00005
14. BSH	16	Bashahe, Lvchun, Yunnan, China	W	22°53′N, 101°20′E	680–960	H1 (16)	0.00000 ± 0.00000	0.00000 ± 0.00000
15. HH	14	Chiang Mai, Thailand	W	18°53′N, 98°51′E	600	H1 (14)	0.00000 ± 0.00000	0.00000 ± 0.00000
16. HTH	10	Chiang Rai, Thailand	W	20°17′N, 99°48′E	580	H1 (10)	0.00000 ± 0.00000	0.00000 ± 0.00000
17. CAM	14	Cambodia	W	12°02′N, 104°27′E	70	H5 (14)	0.00000 ± 0.00000	0.00000 ± 0.00000
18. LP	12	Luang Prabang, Laos	W	19°37′N, 102°11′E	500	H6 (12)	0.00000 ± 0.00000	0.00000 ± 0.00000
19. PU	12	Pumate, Vietnam	W	19°00′N, 104°40′E	10	H1 (2), H9 (10)	0.303 ± 0.147	0.00048 ± 0.00023
20. CHM	12	Chiangmai, Thailand	W	19°15′N, 98°55′E	500	H1 (12)	0.00000 ± 0.00000	0.00000 ± 0.00000
21. WR	12	Wanrong, Laos	W	19°06′N, 102°22′E	400	H1 (12)	0.00000 ± 0.00000	0.00000 ± 0.00000
22. WTS	10	Mengla, Yunnan, China	W	21°37′N, 101°35′E	680	H1 (8), H12 (2)	0.356 ± 0.159	0.00019 ± 0.00008
23. XHJ	12	Xiaoheijiang, Ning'er, Yunnan, China	W	23°21′N, 100°55′E	800	H1 (12)	0.00000 ± 0.00000	0.00000 ± 0.00000
24. YXG	16	Yexianggu, Jinghong, Yunnan, China	W	22°10′N, 100°51′E	760	H1 (16)	0.00000 ± 0.00000	0.00000 ± 0.00000
Total	320						0.734 ± 0.015	0.00094 ± 0.00003

E indicates east of the Tanaka line, W indicates west of the Tanaka line.

Tacca (Dioscoreaceae) is a small genus of tropical herbs with pantropical distribution. Plants of this genus possess near black flowers, conspicuous involucral bracts, and whisker-like filiform bracteoles (Zhang et al., 2011a). *Tacca chantrieri* is an excellent system for evaluating the population genetic structure and phylogeography of herbaceous plants since it is widely distributed throughout the humid tropical forest understory in the Indo-Burma area (Drenth, 1972). *T. chantrieri* inhabits moist and shaded understory habitats usually found along streams or rivers in valleys, and its purplish-black fruits are often consumed by rodents (personal observations). The natural populations of *T. chantrieri* were destroyed in recent years due to human activities, and it is listed as an endangered plant in China (Fu and Jin, 1992).

Using inter simple sequence repeat (ISSR) markers, we previously demonstrated that there is a strong genetic differentiation in *T. chantrieri* across eastern and western parts of the Tanaka line (Zhang et al., 2006), but the divergent time and the factors that resulted in the genetic differentiation of *T. chantrieri* between the two lineages were unclear. Here, we utilize chloroplast DNA variation of *T. chantrieri* distributed in tropical regions to elucidate phylogeographic patterns and demography of this species and then explore the possible causes that can explain the phylogeographic patterns of distribution in *T. chantrieri*.

2. Materials and methods

2.1. Plant materials

A total of 320 samples of *T. chantrieri* were collected from 24 field populations in mainland Southeast Asia, covering most of the distribution range of this herb (Table 1 and Fig. 1). Leaf material was collected at intervals of at least 10 m, and plants were chosen randomly. Young, healthy leaves were collected and immediately dried with silica gel in the field. Depending on the population size, a total of 10–15 individuals from each population were collected. Voucher specimens of the samples were deposited in the herbarium of Xishuangbanna Tropical Botanical Garden, Chinese Academy of Sciences (HITBC), and the Herbarium of Pumate National Park, Vietnam.

2.2. DNA extraction, PCR amplification, and sequencing

Total DNA was extracted using a modified CTAB method (Doyle and Doyle, 1987). Three chloroplast regions, *trn*H-*psb*A, *trn*S-*trn*G, and *trn*L-F, were used to measure variation based on a preliminary screen of seven DNA regions (the other regions

were ITS, *rpo*B-*trn*C, *rps*16, and *rpl*16). Polymerase chain reaction (PCR) amplification and DNA sequencing were performed with universal primers for *trn*L-F (5′-CGAAATCGGTAGACGCTACG-3′ and 5′-ATTTGAACTGGTGACACGAG-3′) (Taberlet et al., 1991), *trn*S-G (5′-AGATAGGGATTCGAACCCTCGGT-3′ and 5′-GTAGCGGGAATCGAACCCGCATC-3′) (Hamilton, 1999), and *trn*H-*psb*A (5′-CGCGCATGGTGGATTCACAATCC-3′ and 5′-GTTATGCATGAACGTAATG

CTC-3′) (Sang et al., 1997). PCR assays were carried out in a total volume of 20 μL containing 20–30 ng template DNA, 5 μL 10 × PCR buffer, 1.5 μL $MgCl_2$ (25 mmol/L), 1 μL dNTP mix (10 mmol/L), 2 μL (5 μmol/L) of each primer, 0.3 μL Taq polymerase (5U/μL) (TaKaRa, Dalian, China)and 9 μL ddH_2O. DNA amplification was performed on a GeneAmp PCR System 9700 thermal cycler (PerkinElmer, Foster City, CA, USA). The PCR protocol for amplification of the *psb*A-*trn*H spacer included the following steps: 94 °C for 5 min, followed by 32 cycles of 52 °C for 1 min and 72 °C for 1 min, and a final extension step at 72 °C for 7 min. The PCR protocol for the *trn*S-G and *trn*L-F spacers included the following steps: 95 °C for 5 min, followed by 34 cycles of 94 °C for 1 min, annealing temperature (50–65 °C, +0.3°C/cycle) for 1 min, and 65 °C for 1 min; and a final extension at 65 °C for 7 min. PCR products were separated on 1% TAE agarose gels and then purified using a Sangon Purification Kit (Sangon, Shanghai, China) following the manufacturer's protocol. Purified PCR products were used for direct sequencing with a PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA, USA), according to the manufacturer's recommendations. The same primers were used for sequencing by Sangon Biotech Co., Ltd. (Shanghai, China).

2.3. Data analysis

2.3.1. Molecular variability and genetic structure of the population

DNA sequences were aligned using Clustal W (Larkin et al., 2007). Sequence ambiguities were resolved by comparing complementary strands. Sequences representing all haplotypes generated from this study were deposited in GenBank (accession numbers KJ476693- KJ476694 for *trn*S-G, KJ476687-KJ476691 for *trn*L-F and KJ476679-KJ476685 for *psb*A-*trn*H; outgroup of *T. subflabellata* accession numbers KJ476695 for *trn*S-*trn*G, KJ476692 for *trn*L-F and KJ476686 for *psb*A-*trn*H). The congruency between the three cpDNA regions was calculated using the incongruence length difference (ILD) test (Farris et al., 1994) function in PAUP* 4.0b10 (Swofford, 2002) before the data were analyzed. The results showed a high degree of homogeneity between the three regions ($P > 0.05$).

Population genetic parameters, including nucleotide diversity (π) and haplotype diversity (H_d), were calculated with DnaSP 4.0 (Rozas et al., 2003). Population diversity (H_S, H_T) and differentiation (G_{ST}, N_{ST}) parameters were estimated using the program Permut 2.0 (http://www.pierroton.inra.fr/genetics/labo/software/permut) with 1000 replicates according to the methods described by Pons and Petit (1996), which indicates the presence of a phylogeographical structure when the N_{ST} value is higher than the G_{ST}, estimate.

Population structure was assessed using the simulated annealing procedure, which was implemented in Spatial Analysis of Molecular Variance (SAMOVA) 1.0 software (Dupanloup et al., 2002). Additionally, to gather geographically homogenous populations to match the user-defined number of groups ($K = 2$–10), the F_{CT} value was determined for every calculation. The configuration with the largest F_{CT} value was retained as the best grouping of populations. Hierarchical analysis of molecular variance (AMOVA) (Excoffier et al., 1992), which was conducted to assess genetic differentiation within and between geographical regions, was performed using ARLEQUIN 3.0 (Excoffier et al., 2005), with significance tested by 1000 permutations.

The relationships among haplotypes were estimated using Network 4.6.0.0, followed by the median-joining method (Bandelt et al., 1999). In the analyses, *T. subflabellata* was used as an outgroup.

2.3.2. Phylogenetic analyses and estimation of divergence time

Phylogenetic trees and divergent times among the 12 haplotypes of *Tacca chantrieri* were estimated using a Bayesian method implemented in Beast 1.5.2 (Drummond and Rambaut, 2007). The dataset was analyzed with a log-normal relaxed clock, the extended Bayesian skyline tree, and the best-fitting F81 substitution model, which was selected using the Akaike information criterion (AIC) as implemented in ModelTest 3.7 (Posada and Crandall, 1998). Given the lack of fossil records for noncoding cpDNA regions of *T. chantrieri*, we assumed a substitution rate of 1.52×10^{-9} substitutions per neutral site per year (s/s/y) (Wolfe et al., 1987). Wolfe et al. (1987) estimated the rate of divergence for cpDNA was $1.0–3.0 \times 10^{-9}$ s/s/y for sites not under selective pressure. Therefore, we set 1.0×10^{-9} s/s/y as the lower limit and 3.0×10^{-9} s/s/y as the upper limit for nucleotide substitution rate in our analyses (Dick et al., 2007; Yuan et al., 2008; Wang et al., 2013).

Three independent Markov chain Monte Carlo (MCMC) analyses were performed for 40,000,000 generations sampling every 4000. Logs and tree files were combined in LogCombiner 1.7.4 (Drummond et al., 2012). Effective sample size (ESS) values and frequency plots were examined using TRACER 1.5 (Rambaut and Drummond, 2009), and trees were summarized in TreeAnotator1.7.4 (Drummond et al., 2012), with 10% burn-in, and displayed in FigTree1.4 (Rambaut, 2012).

2.3.3. Demographic analyses

Mismatch distribution analyses, which represent the frequency distributions of pair-wise nucleotide differences among all haplotypes (Rogers and Harpending, 1992), have been plotted using DNASP 4.0 (Rozas et al., 2003) to test whether *T. chantrieri* had undergone recent demographic population expansion; multimodal mismatch distributions of pair-wise differences between haplotypes are found in populations at demographic equilibrium, whereas unimodal distributions are found in populations that have experienced recent demographic expansions (Rogers and Harpending, 1992; Harpending, 1994).

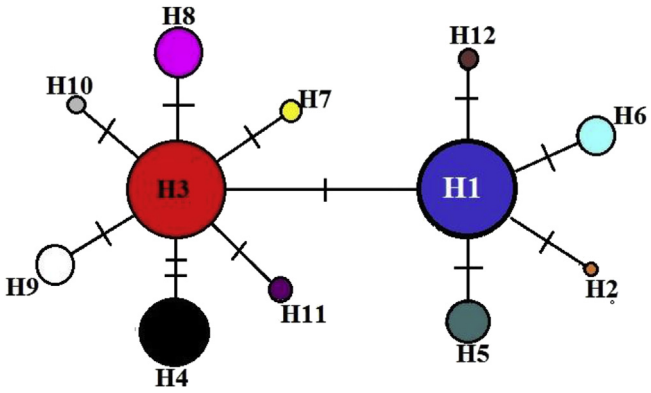

Fig. 2. Statistical parsimony network depicting the 12 haplotype relationships of *T. chantrieri*. Haplotypes are designated by numbers; each line between haplotypes indicates a mutational step. The size of the circle indicates the frequency of each haplotype.

Goodness of fit was evaluated with the sum of squared deviations (SSD) to test the validity of the expansion modal distribution between observed and expected mismatch distributions and with Harpending's raggedness index (H_{Rag}) to quantify the smoothness of mismatch distributions (Harpending, 1994) using 1000 parametric bootstrap replicates implemented in ARLEQUIN 3.0 (Excoffier et al., 2005). Tests of neutrality-Tajima's D (Tajima, 1989) was investigated to detect historical demographic expansions using DNASP 4.0 (Rozas et al., 2003).

2.3.4. Phylogeographical inference using RASP 2.0

The possible ancestral range or the mechanism (vicariance and/or dispersal) underlying the geographically disjunct distribution of *T. chantrieri* were tested by statistical dispersal-vicariance analysis (S-DIVA) and Bayesian binary MCMC (BBM) analysis using RASP 2.0 (Yu et al., 2011) with trees from the Beast MCMC outputs; the Beast annotated tree was set as the final condensed tree. The maximum number of areas at each node allowed in ancestral distributions was set at 3. The other parameters were automatically optimized. In our analysis, two geographical areas were used to describe the distribution of *T. chantrieri* according to the result from SAMOVA analysis (Fig. 1 and Table 1): the left side (west) of the Tanaka line and the right side (east) of the Tanaka line. Outgroup individuals were excluded from the S-DIVA analysis.

3. Results

3.1. Sequence characteristics and genetic diversity

The three cpDNA regions amplified in this study had a combined length of 1897 bp, of which *trn*L-F, *trn*H-psbA, and *trn*S-G made up 852, 318, and 727 bp respectively. Twelve polymorphic sites (0.63%), including eight single variable sites, were detected within the three combined sequences, which were concatenated and treated as one sequence in all analyses. Among them, one polymorphic site (1 polymorphism detected in 852 aligned positions; 0.14%) was detected in the *trn*S-G region, four polymorphic sites (4/727; 0.47%) were detected in the *trn*L-F region (including the *trn*L intron, *trn*L exon, and *trn*L-F spacer), and seven polymorphic sites (7/318; 2.20%) were detected in the *trn*H-*psb*A region. For the cpDNA data, the average within-population diversity ($H_S = 0.097 \pm 0.0312$) was much lower than the total diversity ($H_T = 0.762 \pm 0.0502$). The overall haplotype diversity (H_d) was 0.734 ± 0.015 and the nucleotide diversity (π) was 0.00094 ± 0.00003, which indicates a low level of nucleotide diversity in the whole species.

In total, 12 haplotypes were recovered from the 24 populations. The distributions in each population and frequencies of these haplotypes are listed in Table 1 and Fig. 1. Six haplotypes (H3, H4, H7, H8, H10, and H11) from populations 1–12 were found east of the Tanaka Line, and the other haplotypes (H1, H2, H5, H6, H9, and H12) from populations 13–24 were found west of the Tanaka Line. No haplotypes were shared between the two regions. All haplotypes were distinguished from each other by one or two mutational steps. The statistical parsimony haplotype network of *T. chantrieri* revealed a single network (Fig. 2).

3.2. Population genetic and phylogeographical structure

Analysis of spatial genetic structure for chlorotype variation using SAMOVA revealed that F_{CT} value maximizes to a value of 0.6758 when K was 2 (where K is the number of groups). Therefore, it was appropriate to divide all populations into two groups. The grouping pattern of populations is shown in Fig. 1 and Table 1, where populations found east of the Tanaka line, including populations 1–12, could act as one group, and populations found west of the Tanaka line, including populations 13–24, could act as the other group. The results of AMOVA are presented in Table 2 showed that 67.58% of the total variation

was shared between these two groups, and 26.40% of the variation was shared among populations within the region itself, and only 6.02% of the variation was shared within the populations. Furthermore, AMOVA also showed that a large proportion of the molecular variance could be attributed to genetic differences among populations within the total ($F_{ST} = 0.9398$) and between these two groups ($F_{CT} = 0.6758$). The F_{SC} value also indicated high differentiation among the populations within groups ($F_{SC} = 0.81435$). A test for phylogeographic structure of haplotype variation across the distribution of the species showed that the *N*st value was significantly higher than the G_{ST} value ($N_{ST} = 0.887 \pm 0.034$; $G_{ST} = 0.873 \pm 0.039$, $P < 0.05$), indicating significant phylogeographic structure in *Tacca chantrieri* across the species' range (Pons and Petit, 1996).

3.3. Phylogeographical relationships and divergence time estimates

Relationships among all identified haplotypes (Fig. 3) were very similar to the topology indicated by the haplotypic network (Fig. 2). The phylogenetic relationships among haplotypes of *Tacca chantrieri* were clustered into two clades with high posterior probability (PP) values in the Beast analysis; these were termed clade I and clade II. Clade I included five haplotypes (H1, H2, H5, H6, and H12) that were fixed in populations located west of the Tanaka line. Clade II comprised seven haplotypes (H3, H4, H7, H8, H10, and H11) from 13 populations (QCT, MLP, GLQ, TP, DL, GXNG, PS, CZ, HN, QZ, QXL, WN, and PU), which were located east of the Tanaka line except for population PU. Additionally, similar to the results of the network analysis, haplotype H9 was genetically related to the population found east of the Tanaka line, although it was located in the PU population.

Based on the dating analyses using a substitution rate of 1.52×10^{-9} s/s/y, the divergent time between the east and west region groups was found to be 1.16 Ma (95% highest posterior density [HPD]: 0.44–2.05 Ma; node a; Fig. 3). when using lower and upper nucleotide substitution rates of 1.0×10^{-9} and 3.0×10^{-9} s/s/y, the divergent times between the east and west region populations were estimated at 1.76 Ma (95% HPD: 0.71–3.18 Ma) and 0.61 Ma (95% HPD: 0.23–1.07 Ma) respectively. Based on these results, the divergence of population east and west of the Tanaka line in *T. chantrieri* is estimated to have occurred in the mid-Pleistocene period.

3.4. Demographic history and historical biogeography inference

The cpDNA sequence mismatch analysis results for all *Tacca chantrieri* populations and for each group displayed a multimodal distribution pattern that differed from the predicted pattern under a model of sudden range expansion. This difference was also supported by tests of neutrality, which showed non-significant values in Tajima's D for all *T. chantrieri* populations and for each group. The results indicated that no sudden population expansions occurred during most of the history of this species. However, statistical comparisons (SSD and H_{Rag}) showed a good statistical fit between these observed distributions and simulated ones under a sudden (stepwise) demographic expansion model (SSD: all populations:0.026, East of Tanaka line: 0.042, West of Tanaka line:0.011, $P > 0.05$; H_{Rag}:all populations: 0.158, East of Tanaka line:0.190, West of Tanaka line: 0.126; $P > 0.05$). Ancestral ranges obtained by RASP 2.0 analysis are shown in Fig. 3. There were two vicariance events and one dispersal event in total. The results supported a distinctive vicariance event (node a, Fig. 3) for *T. chantrieri* between populations east and west of the Tanaka line. The populations of *T. chantrieri* experienced both vicariance (node a, b) and long-distance dispersal (node c), which indicated limited gene flow between east and west of the Tanaka line.

4. Discussion

4.1. Strong genetic differentiation and limited gene flow

High genetic differentiation ($N_{ST} = 0.887$, $G_{ST} = 0.873$) was observed within *T. chantrieri* when compared with the mean G_{ST} values (0.637) detected from other angiosperm species (Petit et al., 2005). AMOVA analysis provided similar evidence, which revealed that 26.40% of genetic variation occurred among populations and 6.02% within populations (Table 2). The results of this study were consistent with our previous study using ISSR markers (Zhang et al., 2006). Given the characteristics and habitat of the species, four reasons are proposed to explain these results. First, we expect the genetic variation to be higher among populations than within populations since most *T. chantrieri* seeds result from autonomous self-pollination (Zhang et al., 2005). Such patterns are consistent with those found in other self-pollinated species (Hamrick and Godt, 1996).

Table 2
Hierarchical Analysis of molecular variance (AMOVA) between populations of *T. chantrieri* based on sequences of cpDNA *trn*L-F, *trn*S-G, and *trn*H-*psb*A regions.

Source of variations	d.f.	s.s.	v.c.	p.v.	Fixation index
Among groups	1	151.205	0.91373	67.58	$F_{CT} = 0.67581$, $p < 0.001$
Among populations within groups	22	106.148	0.35694	26.40	$F_{ST} = 0.93981$, $p < 0.001$
Within populations	296	24.087	0.08138	6.02	$F_{SC} = 0.81435$, $p < 0.001$
Total	319	281.441	1.35206		

d.f., degrees of freedom; s.s., sum of squares; v.c., variance components; p.v., percentage of variation.

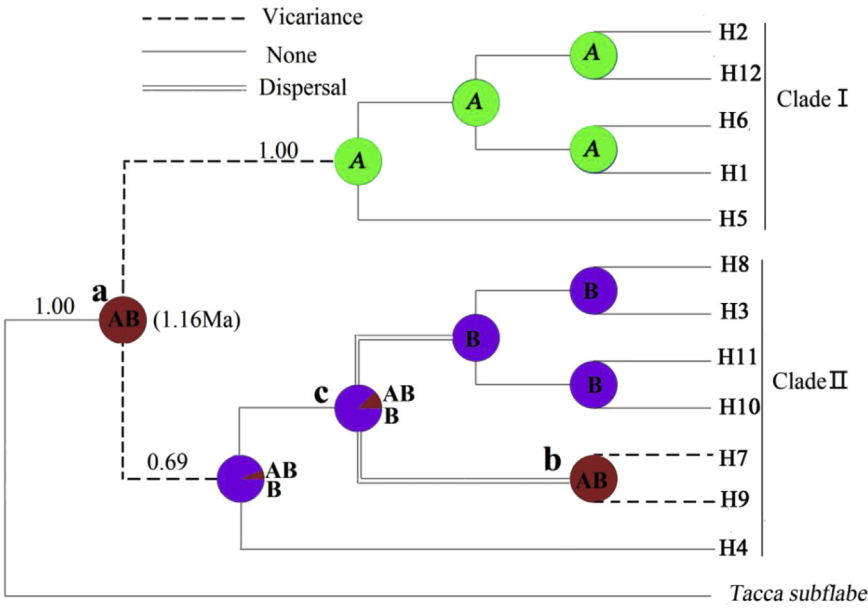

Fig. 3. Phylogenetic relationships of the cpDNA haplotypes of *T. chantrieri* generated by Beast. Posterior probabilities are shown above the branches. Pie charts indicate the proportion of the ancestral ranges based on RASP 2.0 analysis. The distribution areas of extant populations of *T. chantrieri* are marked by A and B. Lower case letters (a–c) indicate nodes discussed in the text. The double lines and dashed straight lines indicate the long-distance dispersal (LDD) and vicariance events, respectively. Numbers in brackets are the estimated divergent times million years ago.

Second, although good dispersal ability could enhance both the ability of range change and adaptive evolution (Lavergne et al., 2010), the heavy fleshy fruits of *T. chantrieri* are dispersed mainly by gravity, thus, shortening the average seed dispersal distance and hence limiting gene flow among populations. Third, a mosaic of plateaus, mountains, basins and gorges were created through the Himalayan movements during the early Pleistocene in Yunnan and its surrounding areas (Committee of Chinese Academy of Sciences for Physical Geography of China, 1984) creating barriers to movement and thus causing limited gene flow among populations. Lastly, human activities such as overharvesting of the species and extending the cultivated area in recent years resulted in a dramatic reduction in population size in the wild, which could influence the genetic differentiation of *T. chantrieri*.

4.2. Patterns of geographic structure and historical biogeography inference

In this study, there was obvious vicariance between clade I and clade II from the S-DIVA analysis, because cpDNA data revealed a strong phylogeographical structure in *T. chantrieri* populations and the divergence of the *T. chantrieri* populations between two clades occurred 1.16 Ma (95% HPD: 0.44–2.05 Ma) when applying substitution rates of 1.52×10^{-9} s/s/y, at the same time, the divergent time was between 0.61 Ma (95% HPD: 0.23–1.07 Ma) and 1.76 Ma (95% HPD: 0.71–3.18 Ma) when using Substitution rates of $1.0–3.0 \times 10^{-9}$ s/s/y, both of the divergent time occurred during the mid-Pleistocene. Thus, there was a distinctive vicariance between populations east and west of the Tanaka line as a whole in terms of S-DIVA and BBM analyses. Hence, we put forward two reasons—the red river as a barrier and the monsoon climatic barrier associated with the Tanaka line—to explain the divergence of *T. chantrieri*.

First, as is the case in most species (Hewitt, 2000), we propose that the distribution and genetic diversity of *T. chantrieri* was also affected by cycles of fragmentation and range expansion with the Red River acting as a barrier for genetic differentiation. Based on the divergent time of *T. chantrieri* and the climatic changes that occurred during the Quaternary, *T. chantrieri* was considered to experience a north-south migration during the Quaternary to escape from severe cold and drought triggered by glaciers. In fact, the substantial phylogeographic break between the 'western' and 'eastern' lineages of *T. chantrieri* occurs along the Red River region (Fig. 1), which means that a small distributional gap may exist between the two lineages through the Red River. Therefore, the Red River between the two sides of the Tanaka line, which begins in China's Yunnan province in the mountains south of Dali and flows generally southeastward to enter Vietnam at Lào Cai Province, probably acts as a topographical east-west barrier severely hindering the gene flow between two regions. But it is worthwhile to note that the PU population was unique because it possessed both a 'western' haplotype (H1) and an 'eastern' haplotype (H9). We consider two hypotheses to explain the genetic characteristics of the PU population. (1) there is a recent westward expansion with one-step mutation (from H3 to H9) by accident of the individuals from eastern clade into the western site. (2) the H9 population, which was derived from H1 through 2-step mutations, was fixed in the PU population due to several possible population characteristics such as small population size, inbred mating patterns or genetic drift.

The second factor that can explain the divergence of *T. chantrier* is the climatic monsoon characteristics associated with the Tanaka line, which demarcates the subdivision of East Asia into the Sino-Japanese in the east and Sino-Himalayan in the west. The Tanaka line was first suggested by the Japanese scholar Tyôzaburô Tanaka (Tanaka, 1954) in the taxonomic classification and distribution of Citrus. Li and Li (1997) implied that a combination of historical (tectonic) and ecological (climate-related) processes have resulted in the formation of the Tanaka line. However, opinions regarding the validity of this assertion differ. Fan et al. (2013) concluded that the tectonic and climate-related processes were unlikely to have been initiated simultaneously because the time of divergence between *Sophora davidii* populations on the two sides of the Tanaka line coincides with the climate-related events, which was established during the Pleistocene (Wang, 1994; Fort, 1996; Mitsui et al., 2008) rather than tectonic events (initiated from Tertiary/mid-Miocene to Late Pliocene) (Royden et al., 2008; Li et al., 1979; Zhou et al., 2006). In our study, we agree with Fan et al. (2013), not only because the divergent time of *T. chantrieri* (1.16Ma) was similar with the *S. davidii* (1.28Ma), but also because there exists different monsoon climates (southwest [SW] monsoon vs. southeast [SE] monsoon) in the distribution area of *T. chantrieri*. However, there are different opinions about the boundary of the two monsoon climate. For example, Zhang (1982) indicates that Sichuan province and east part of Yunnan, in which the wind direction changes unclearly between winter and summer, was the dividing line between the SW and SE monsoons and Gao et al. (1962) points out that the east part of Yunnan, which lies between the two precipitation types, was influenced alternately by the monsoons of SE and SW. In addition, Wu et al. (2012) consider that the boundary was changed in different months due to the barrier-corridor effect of longitudinal range-gorge terrain. Hence, taking recent divergent time and the distribution area into account, it is possible that the different monsoon climates, which estimated to have occurred during the late Pleistocene (Fort, 1996) or the late Pliocene and Pleistocene (Hsü, 1978), may in part influence the phylogeography of *T. chantrieri* by acting as an ecological barrier in Yunnan and adjoining area, and the Tanaka line, which split a west-east divergence in *T. chantrieri*, is considered to be associated with the different monsoon climate. To clarify the boundary line between the SE monsoon and the SW monsoon, more climatic, geographical and molecular phylogeographic proof in future analysis of other species in this region is needed.

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