JSE Journal of Systematics and Evolution

Journal of Systematics and Evolution 50 (1): 45–57 (2012) doi: 10.1111/j.1759-6831.2011.00166.x

Research Article

Population differentiation and phylogeographic pattern of a relict species, *Conandron ramondioides* (Gesneriaceae), revealed from sequence polymorphism and haplotypes of the *CYCLOIDEA* gene

[1,2]Li-Hong XIAO [1]Zhi LI [3]Rui WANG [2]Yin-Zheng WANG*

[1](*College of Life Science, Capital Normal University,* Beijing 100037, China)
[2](*State Key Laboratory of Systematic and Evolutionary Botany, Institute of Botany, Chinese Academy of Sciences,* Beijing 100093, China)
[3](*State Key Laboratory for Biology of Plant Diseases and Insect Pests, Institute of Plant Protection, Chinese Academy of Agricultural Sciences,* Beijing 100094, China)

Abstract Historical events are expected to affect population genetic differentiation and DNA molecular evolution, but the impact of these effects remains a matter of debate. Here, for *Conandron ramondioides* (Gesneriaceae), we analyzed the genetic structure and phylogeographical pattern of 248 individuals from 13 populations, distributed in mainland China and Taiwan Island, based on the nucleotide sequence and haplotype of the coding sequence of *CYCLOIDEA1* (*GCYC1*). Among the populations, we found a high level of haplotype diversity ($h = 0.831$) and a relatively low level of nucleotide diversity ($D_{ij} = 0.004$). Both the haplotype network and the neighbor-joining tree constructed from *GCYC1* haplotypes suggest two major geographical groupings, one on the mainland and the other on Taiwan. Consistently, AMOVA analysis revealed high genetic differentiation between these groupings, with 84.65% variation partitioning the two regions, and the two groupings shared no haplotype. On the mainland, population genetic differentiation was correlated with more recent events, presumably Pleistocene glaciations and human activities since the Neolithic. In addition, *C. ramondioides GCYC1* rapidly accumulated neutral mutations, consistent with this gene being silenced or down-regulated in actinomorphic lineages of the Lamiales, such as *Conandron.*

Key words *Conandron ramondioides*, genetic diversity, geographical isolation, haplotype, phylogeography.

As argued by Wright (1965) and Nei (1975), each species distributes its genetic diversity in a pattern reflecting both its biology and its history. Relevant historical factors include geological and climatic changes (i.e., island formation and glaciation) and, more recently, human-induced disturbances (fragmentation, global change). For example, current patterns of genetic diversity in *Ramonda myconi* (L.) Rchb., a relict species of Gesneriaceae from northeastern Spain, suggest that several historical factors acting throughout the Pleistocene have been decisive in shaping both its genetic structure and species distribution (Picó & Riba, 2002; Picó et al., 2002; Dubreuil et al., 2008). Another case is *Titanotrichum oldhamii* (Hemsl.) Soler. (Gesneriaceae), in which strong genetic differentiation is found between the populations from Taiwan and Fujian provinces in China (Wang et al., 2004a, 2004b), presumably because of island formation 5 Mya (Shen, 1994). Organelle genetic variation of *Picea jezoensis* Carr. var. *microsperma* (Lindl.) Cheng & L. K. Fu, distributed in northeast Asia, revealed different mitochondrial haplotypes between Hokkaido and Honshu islands. The differences might be associated with the separation of these islands from each other and from the Asian continent during the late Quaternary (Aizawa et al., 2007).

Conandron ramondioides Siebold & Zucc. is a tertiary relict plant species that constitutes a monotypic genus, phylogenetically distinct from other taxa in the old world Gesneriaceae (subfamily Cyrtandroideae) (Burtt, 1963; Wang et al., 1990; Wang, 2004; Weber, 2004). This perennial and rhizomatic herb with only a single aerial leaf is mainly distributed in central, eastern, and southeastern China and is also found in Taiwan Island and Japan. Its local populations are usually small and restricted to isolated mountain areas. Therefore, its populations are highly fragmented and isolated (Wang, 2004; Xiao & Wang, 2007). However, the habitats are similar; these plants are usually distributed in mountain valleys at middle to low elevation, and they grow on wet, dripping and moss-covered granite rocks under subtropical, evergreen, broad-leaf canopies (Xiao & Wang, 2007).

Received: 30 May 2011 Accepted: 18 August 2011
* Author for correspondence. E-mail: wangyz@ibcas.ac.cn; Tel.: 86-10-62836474; Fax: 86-10-62590843.

Previous research on the molecular phylogeny of *C. ramondioides* shows that there is evident genetic differentiation among some of the populations in China (Xiao & Wang, 2007). However, sampling was limited to populations in southeastern China, which is insufficient to identify the major evolutionary and historical processes that have shaped the present geographic distribution of this species. It remains unclear what genetic relationship exists between the populations from Taiwan Island and the mainland, and how the Taiwanese populations have evolved following island formation, which occurred approximately 9 Mya and attained their current flora approximately 5 Mya (Shen, 1994; Chiang & Schaal, 2006).

Conandron is believed to represent a derived actinomorphic lineage early in the diversification of Gesneriaceae, which is consistent with its flower morphology, for which no trace of zygomorphy has apparently ever been observed (Wang, 2004). In genetic models and natural populations, reversal from zygomorphy to actinomorphy is widely associated with the loss-of-function or down-regulation of *CYCLOIDEA* (*CYC*) and its homologues (Luo et al., 1996, 1999; Cubas et al., 1999a, 1999b; Cubas, 2004; Zhou et al., 2008). *CYC* is a key regulatory gene for flower morphology in many lineages (Luo et al., 1996, 1999). The homologue of *CYC* in the Gesneriaceae, *GCYC1*, has been reported to be under neutral selection in a small number of Chinese populations of *C. ramondioides* (Xiao & Wang, 2007); however, it is of interest to find how this regulatory gene has evolved over the longer period since the current Asian populations were separated. It is also interesting to determine whether *GCYC1* in the Taiwanese populations is under neutral evolution or under other selective factors related to island formation.

We further explored the genetic structure and phylogeographical pattern of 13 populations of *C. ramondioides* distributed in Taiwan Island and central to southeast China, based on haplotype data from the coding sequence of *GCYC1*. Our specific aims were to: (i) elucidate the genetic variation between and within populations and among geographic regions in *C. ramondioides*; (ii) try to find any haplotype structure characteristics of unique-event polymorphism mutations correlated with potential historical events, such as island formation, Quaternary glacial impact, and anthropogenic activities; (iii) identify the main evolutionary and historical processes that have shaped the geographic and genealogical patterns in this species; and (iv) further explore how the *Conandron GCYC1* has evolved in the long evolutionary history of *Conandron* populations that were fragmented in different historical periods consequent upon different geological events, which would provide new insight into the molecular evolution of protein-coding sequences under the condition of loss-of-function or down-regulation.

1 Material and methods

1.1 Plant materials

For this research, we carried out field investigations from July 2003 to July 2009. We defined five geographical regions (Fig. 1, Table 1) according to topography. Some populations of *C. ramondioides* recorded in herbaria, such as those in Jing'an and Xiushui in Jiangxi province, had become extinct, probably due to deforestation and other human activities in recent decades (Fig. 1). A total of 248 *Conandron ramondioides* individuals were collected from 13 wild relict populations distributed in Taiwan, Anhui, Zhejiang, and Fujian provinces in central to southeastern China over 7 years (Fig. 1; Table 1). Most of the samples (207 of 248) used in this study were derived from the earlier publication by Xiao & Wang (2007). Seventeen samples from QS21 were re-collected due to the small number of samples presented by the previous study. Samples from NT01 and HS02 were obtained in July 2009. Young and healthy fresh leaves were collected from at least 16 randomly chosen individuals for each population, as described previously (Xiao & Wang, 2007), except for the Taiwan population (NT01) for which only five individuals were sampled because of the difficulty of collection. Some living plants, rhizomes, and seeds, were brought back to the Institute of Botany, Chinese Academy of Sciences (IBCAS) and grown in a glasshouse for future studies. Voucher specimens were deposited in the China National Herbarium (PE).

1.2 DNA extraction, polymerase chain reaction (PCR) amplification, and DNA cloning and sequencing

Genomic DNA of silica gel-dried leaves was extracted using a modified CTAB method from 41 individuals of HS02, QS21, and NT01 populations as well as seven individuals that previously produced sequences with double peaks (Xiao & Wang, 2007). DNA purified by a Genomic DNA Rapid Purification Kit (Bio Development International Inc, Andover, MA, USA) was used for amplification of the *Conandron GCYC1* gene, using the specific primers GC1 (5′-ATG TTT GGC AAG AAC TCG TAC C-3′) and GcycR1 (5′-CAT TGA CAT TAA GAG ATG GGA G-3′) (Xiao & Wang, 2007). In order to avoid variations caused by PCR, the *GCYC1* gene sequence for each sample was amplified at least twice and sequenced from both forward and reverse directions.

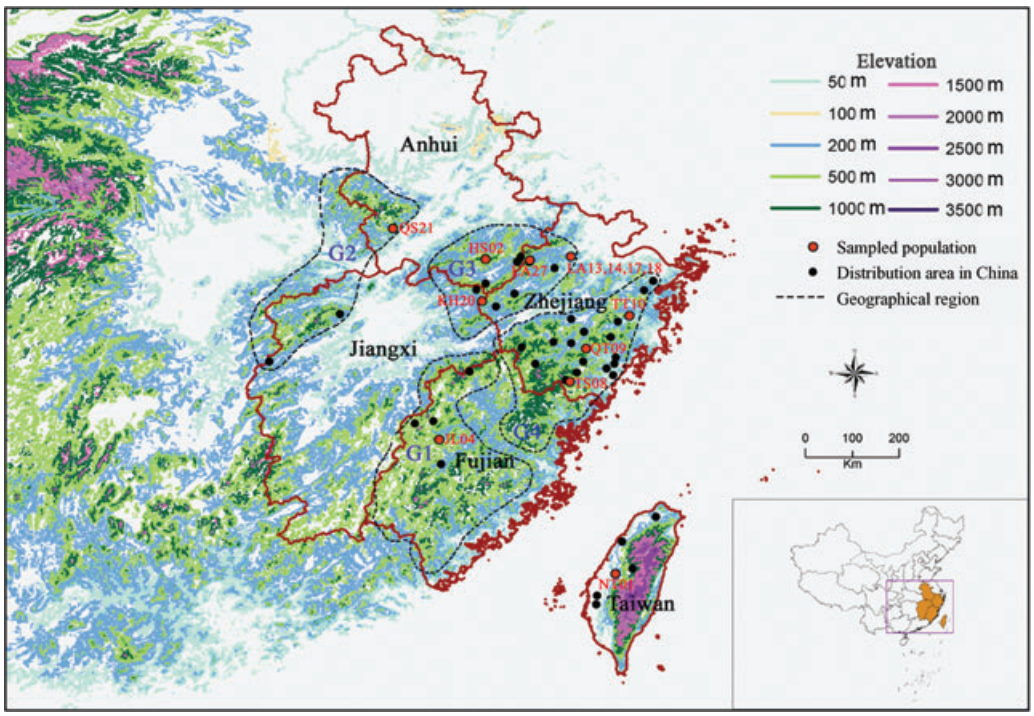

Fig. 1. Topographical map of southeast China. Sampled populations of *Conandron ramondioides* shown as red dots; black dots represent extinct populations. The five defined geographical regions are outlined with a dashed blue line (map from the National Fundamental Geographic Information System, http://nfgis.nsdi.gov.cn/nfgis/chinese/c_xz.htm). G1, Mount Wuyishan; G2, Mountains in western Anhui and Jiangxi provinces; G3, Mount Huangshan, mountains in western Zhejiang province and eastern Jiangxi province; G4, Low mountains and hills in eastern–southern Zhejing province (Tiantaishan–Kuocangshan–Donggongshan).

Table 1 Details of sampling locations, population sizes, and geographical regions of 13 sampled populations of *Conandron ramondioides*

Geographical regions (all in China)	Population and location (all in China)	Population size	Alt. (m)	*n*	Collection time
Mount Wuyishan (G1)	JL04 (Longqishan, Jiangle, Fujian)	253 in 11 m^2	940	21	June 2004
Mountains in western Anhui and Jiangxi provinces (G2)	QS21 (Tianzhushan, Qianshan, Anhui)	48 in 5 m^2	940	17	August 2008
Mount Huangshan, mountains in western Zhejiang province and eastern Jiangxi province (G3)	LA13 (Longtangshan, Lin'an, Zhejiang)	42 in 4 m^2	730	17	July 2004
	LA14 (Longtangshan, Lin'an, Zhejiang)	24 in 3 m^2	720	19	July, 2004
	LA17 (Niulangping, Lin'an, Zhejiang)	120–160 in 8 m^2	1140	32	July 2004
	LA18 (Dong'aotou, Lin'an, Zhejiang)	170 in 10 m^2	960	23	July 2004
	LA27 (Shunxi, Lin'an, Zhejiang)	40 in 5 m^2	510	16	October 2003
	HS02 (Huangshan, Anhui)	40 in 2 m^2	1050	19	June 2009
	KH20 (Gutianshan, Kaihua, Zhejiang)	80–100 in 10 m^2	730	21	July 2004
Low mountains and hills in eastern–southern Zhejing province (Tiantaishan–Kuocangshan–Donggongshan) (G4)	TS08 (Fenghuangshan, Taishun, Zhejiang)	80 in 8 m^2	740	17	October 2003 and July 2004
	QT09 (Kuocangshan, Qingtian, Zhejiang)	140 in 4 m^2	780	19	July 2004
	TT10 (Tiantaishan, Tiantai, Zhejiang)	40 in 4 m^2	760	22	June 2004
Taiwan Island	NT01 (Fenghuangshan, Nantou, Taiwan)	180–200 in 10 m^2	1050	5	August 2005

Alt., Altitude; *n*, Number of sampled individuals.

DNA amplification was carried out in a T1 thermocycler (Biometra, Goettingen, Germany), programmed for an initial 5 min at 94 °C, followed by 32 cycles of 1 min at 94 °C, 1 min at 53 °C, 90 s at 72 °C, and a final 10 min at 72 °C. Reactions were carried out in a volume of 25 μL containing 0.5 μL (5 U/μL) TransStart FastPfu DNA polymerase, 2.5 μL 10× PCR buffer, 2.5 μL 2 mmol/L dNTP, 1.0 μL 25 mmol/L $MgCl_2$, 2.5 μL 2 μmol/L primer (all Beijing TransGen Biotech, Beijing, China), and approximately 50 ng template DNA.

The PCR products were separated by 1.5% (w/v) agarose gel electrophoresis. The desired band was cut out and purified using the EZNA Gel Extraction Kit (Omega Bio-Tek, Norcross, GA, USA). Sequencing reactions were carried out with the forward or reverse primers of the amplification reactions using the DYEnamic ET Terminator Kit (Amersham Pharmacia Biotech, Uppsala, Sweden), following the manufacturer's protocol. Sequencing was carried out on an ABI 3730XL automatic sequencer (Applied Biosystems, Foster City, CA, USA) after the reaction product was purified through precipitation with 95% ethanol and 3 mol/L sodium acetate (pH 5.2). To avoid errors from the PCR and its impact on the observed pattern and level of nucleotide polymorphisms, re-amplification and re-sequencing were carried out for each sample.

We identified heterozygotes by double peaks of the same intensity from the electropherograms. To genotype heterozygotes, the sequences containing double peaks were cloned into pGEM-T Easy plasmids (Promega, Madison, WI, USA); at least five independent clones per sequence with double peaks were obtained for each fragment. Purified plasmid DNA was sequenced in both directions by standard methods on an ABI 3730XL automated sequencer (Applied Biosystems) using universal primers SP6 and T7 (Life Technologies Corporation, Invitrogen, Carlsbad, CA, USA).

1.3 Data analysis

For each individual, the sequences were corrected with Contig Express software (Invitrogen) and aligned using the ClustalX program, version 1.81 (Thompson et al., 1997). Length variation and nucleotide composition were calculated manually using BioEdit version 5.0.6 (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). All haplotype sequences of *GCYC1* were deposited in GenBank, Accession Nos. HQ691255–HQ691295.

We calculated the number of segregating sites (*S*), haplotype diversity (*h*), and nucleotide diversity (*Dij*) at population and species levels (Rozas et al., 2003). To evaluate the intraspecific evolution, we calculated the gene flow and genetic differentiation among populations and a pairwise mismatch distribution to test for population expansion. These parameters were calculated using DNASP 4.50.3 (Rozas et al., 2003) based on coalescent theory. Tests of neutrality were carried out following Tajima (1989)'s *D* as well as Fu & Li (1993)'s D^* and F^*. Recombination rate is an important parameter affecting DNA polymorphism. Therefore, we estimated *C*, the population's recombination rate per site (Hudson, 1987) and *Rm*, the minimum number of recombination events (Hudson & Kaplan, 1985). A neighbor-joining (NJ) cladogram of haplotypes and Kimura 2-parameter distance were calculated using MEGA version 3.0 (http://www.megasoftware.net/).

To assess geographical associations of haplotypes and infer the phylogeographical pattern of *C. ramondioides*, a haplotype network was constructed with the aid of TCS version 1.21: a computer program to estimate gene genealogies from DNA sequences, also known as statistical parsimony (Clement et al., 2000). This method determines the limits of parsimony based on coalescence theory (Hudson, 1990), and uses maximum parsimony to define a set of plausible connections among haplotypes that have >95% confidence intervals (Templeton et al., 1992). Haplotypes were then organized into a network where mutational steps correspond to evolutionary time (Templeton et al., 1992). In the haplotype network, we defined two sets of haplotypes for inferring the phylogeographical pattern of *C. ramondioides*. An UPGMA tree was calculated according to genetic distance Kxy between 13 populations of *C. ramondioides* (Table S1) by MEGA.

To estimate the genetic structure in *C. ramondioides*, we deduced the analysis of molecular variance (AMOVA) by Arlequin 1.1, partitioning into three levels: (i) between Taiwan and mainland China or among four geographic regions on the mainland; (ii) among populations in Taiwan and the mainland or among populations on the mainland; and (iii) within all populations of *C. ramondioides* or within all populations on the mainland.

All of the above programs are aimed at estimating long-term gene flow among populations, as is usually done using the *Fst/Nm* method (Wright, 1951). Wilson & Rannala (2003) have developed a non-equilibrium Bayesian method (BayesAss+) for estimating rates of recent migration among populations, which is a complementary approach and does not require the loci to be in Hardy–Weinberg equilibrium. Also, rather than simply assigning individuals based on population allele frequencies, BayesAss+simultaneously estimates population migration rates, individual migrant ancestries, and the population allele frequencies. This has the important effect of avoiding the bias of including immigrant individuals in the estimation of allele frequencies and is appropriate for the analysis of allelic data including allozymes, microsatellites, restriction fragment length polymorphisms, and single nucleotide polymorphisms. In this study, Bayesian assignment tests were carried out using BayesAss+version 1.3 (Wilson & Rannala, 2003).

To further access correlations between population genetic differentiations and geographic isolation subsequent upon historical events, the putative genetic divergence time between geographical regions was

calculated, aided by MEGA version 3.0, based on sequence data and on the assumption that the divergence time between populations from Taiwan and mainland China is approximately 5 million years (Shen, 1994).

2 Results

2.1 Sequence characteristics and neutral tests

A total of 248 aligned sequences were 831 bp in length. Nucleotide substitutions occurred at 36 sites (Table S2). Single nucleotide change was high, at an average rate of one polymorphism per 23 bp. All 36 nucleotide changes were located in protein-coding regions of *GCYC1* and 28 of them resulted in amino acid substitution. In these sequences, the GC content was moderate, namely 42%. Interestingly, 12 of the 36 nucleotide changes belonged to the Taiwan population, NT01.

Tests of neutrality of *GCYC1* were carried out at the levels of population, geographical region, and species. All but four tests showed non-significant deviation from expectations of neutrality by both Tajima's criterion and Fu and Li's test (Table 2), suggesting that the *GCYC* gene was under little if any selection.

Table 2 Summary of results of neutrality tests at the *Conandron ramondioides GCYC1* locus in populations, geographical regions, and species

		Tajima's D	Fu & Li's D^*	Fu & Li's F^*
Population level	NT01	0.015	0.804	0.684
	TT10	−1.334	−0.665	−1.009
	TS08	1.695	1.307	1.679*
	LA27	0.193	1.138	0.997
	LA18	0.026	0.551	0.463
	LA17	−0.896	0.523	0.125
	LA14	0.704	0.922	0.515
	LA13	−1.326	1.044	0.399
	KH20	−0.947	1.309*	0.698
	HS02	−1.377	1.033	0.363
	QT09	0.133	1.259	1.065
	QS21	−0.400	1.405	0.984
	JL04	0.316	0.559	0.566
Geographical region level	G1	0.316	0.559	0.566
	G2	−0.400	1.405	0.984
	G3	−0.986	1.547*	0.682
	G4	0.379	0.644	0.654
	TW	0.015	0.804	0.684
Species level		−1.153	2.200**	0.840

G1, Mount Wuyishan; G2, Mountains in western Anhui and Jiangxi provinces; G3, Mount Huangshan, mountains in western Zhejiang province and eastern Jiangxi province; G4, Low mountains and hills in eastern–southern Zhejing province (Tiantaishan–Kuocangshan–Donggongshan); TW, Taiwan. HS02, Huangshan, Anhui; JL04, Longqishan, Jiangle, Fujian; KH20, Gutianshan, Kaihua, Zhejiang; LA13, Longtangshan, Lin'an, Zhejiang; LA14, Longtangshan, Lin'an, Zhejiang; LA17, Niulangping, Lin'an, Zhejiang; LA18, Dong'aotou, Lin'an, Zhejiang; LA27, Shunxi, Lin'an, Zhejiang; NT01, Fenghuangshan, Nantou, Taiwan QS21, Tianzhushan, Qianshan, Anhui; QT09, Kuocangshan, Qingtian, Zhejiang; TS08, Fenghuangshan, Taishun, Zhejiang; TT10, Tiantaishan, Tiantai, Zhejiang. Statistical significance: $P > 0.1$; *$0.1 > P > 0.05$; **$P < 0.02$.

2.2 Haplotype diversity and nucleotide diversity

Thirty-six nucleotide substitutions of *GCYC1* resulted in 41 haplotypes (Table S2). No haplotype was shared by all sampled populations. Hap7 and Hap5 were the most common haplotypes, present in 10 and 8 populations, respectively. NT01 (Taiwan) had two unique haplotypes (Hap36, Hap37), whereas QS21 and TS08 had the richest set of haplotypes (10). QS21 had the richest set of unique haplotypes, namely five (Hap29–Hap34).

A high level of haplotype diversity ($h = 0.83147 \pm 0.014$) and a relatively low level of nucleotide diversity ($D_{ij} = 0.00386 \pm 0.00024$) were detected within the species (Table S3). Across all populations, haplotype diversity and nucleotide diversity ranged from 0.06151 to 0.8699, and 0.00007 to 0.00256, respectively. Haplotype diversity showed nearly the same trend as nucleotide diversity in all populations (Tables S2, S3). At the population level, haplotype diversity in QS21 was nearly the same as that in TS08, whereas its nucleotide diversity was lower than the corresponding value of TS08.

2.3 Intraspecific cladogram and phylogeographical inferences

The haplotype network of *GCYC1* divided into two distinct clades, consistent with a significant geographic differentiation between populations from Taiwan and mainland China (Fig. 2, Table S2). The two clades were connected by 15 mutations, suggesting a long and independent evolutionary history. The first clade, with two unique haplotypes (Hap36, Hap37), specifically belonged to the population on Taiwan Island (NT101). The second clade included the remaining haplotypes and involved all of the populations in central to southeastern China. Two haplotypes (Hap5, Hap7) served as central hubs for the clade, from which all of the mainland haplotypes emanated. Eleven haplotypes coalesced to Hap5 by one mutational step and 14 others were linked by two steps. Similarly, six haplotypes were linked to Hap7 by one mutational step and four by two steps. The presence of several loops in the haplotypes present in G3 and G4 geographical regions suggested the existence of recombination between genes (Fig. 2). However, the recombination test did not show significant biased results for neutral tests at species level (Table S4), so the presence of reticulation in the network could be best explained by the homoplasious sites between sequences within populations or from close local populations in the same sampling area (Fig. 1).

Consistent with the haplotype network described above, cluster analysis using an NJ algorithm also identified two distinctive groups (Fig. 3). The Taiwan population (NT01) formed a group of its own, and the 12

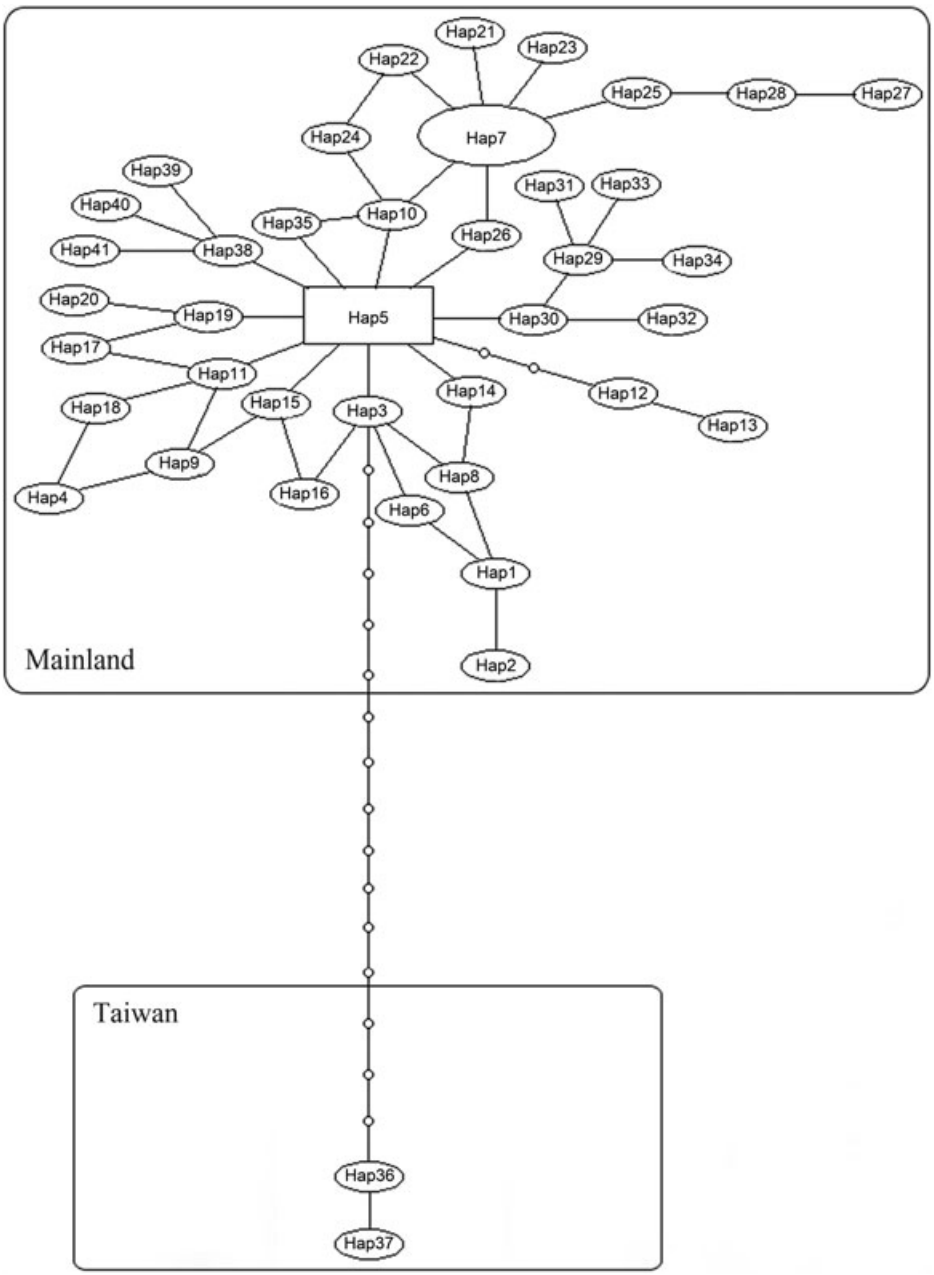

Fig. 2. *GCYC1* haplotype network. Large circles and rectangles represent the indicated haplotype (Hap). Small circles represent inferred interior nodes that were absent from the samples.

mainland populations formed a distinct group. Within the mainland group, only 10 haplotypes (approximately 25%) were present in more than one population (shown in green in Fig. 3). This provides evidence for limited gene flow. Haploytype clusters overlay geography for clusters from Anhui (blue and pink in Fig. 3) but other clusters contained haplotypes from various populations, which is consistent with both gene flow between these populations and selection acting on these haplotypes.

To explore the phylogenetic relations further, the *GCYC1* haplotypes were used to build a universal phylogenetic genealogy with multiple *GCYC1* sequences. An UPGMA tree was also established based on genetic distance between populations from haplotype of *GCYC1* (Fig. 4, Table S1). Consistent with the haplotype network and NJ tree, the UPGMA tree also divided into two main groups, a Taiwan group and a mainland China group (Fig. 4). In the China group, the most divergent

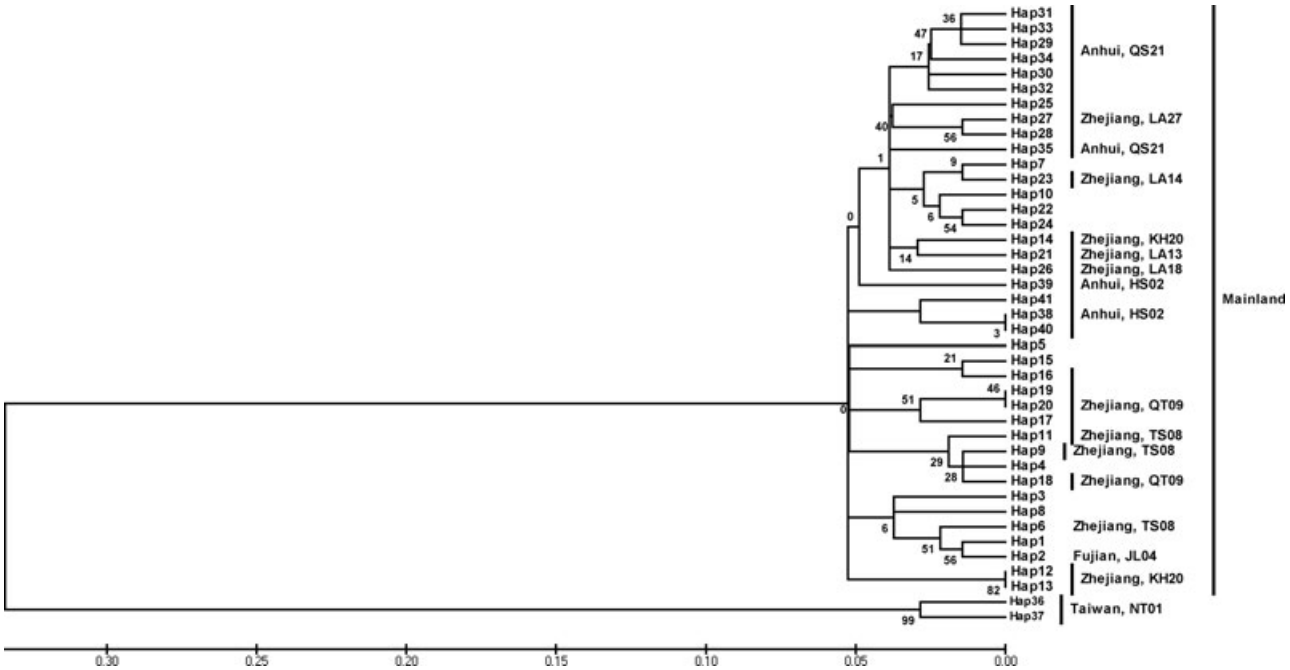

Fig. 3. Neighbor-joining tree of *Conandron ramondioides GCYC1* haplotypes (Hap), showing provinces in mainland China and Taiwan island where populations were collected and sample identifying numbers.

population was from Jiangle (JL04; in Fujian province), which is geographically the most isolated. The populations from Lin'an were monophyletic and sister to another sub-clade containing the populations from Qingtian (QT09), Tiantai (TT10), and Taishun (TS08), and more distantly related to the populations in Huangshan (HS02) and Kaihua (KH20). Insofar as the latter were grouped in the same geographical region as those in Lin'an (G3; Table 1), and the former placed in a separate region (G4), the divisions made in Table 1 appear to miss the salient features of population structure. JL04 was relatively isolated and was sister to all of the above groups.

2.4 Population genetic structure

The population structure of *C. ramondioides* was assessed based on variation of the *GCYC1* coding sequence. Significant genetic differences existed between all 13 populations and between the five geographical regions, as shown by the estimation of both the mean genetic differentiation between populations (*Fst*) (Table 3) and of the gene flow between populations or geographical regions ($Nm = 0.06$ or $Nm = 0.04$). Pairwise *Fst* values among regions and populations reported in Table 3 range from 0.138 to 0.697 and 0.024 to 0.989, respectively. The *Fst* values indicated that most of the observed genetic diversity was due to variation between *C. ramondioides* populations and between geographical regions as the variation that provided by AMOVA (Table 4). The relatively low *Nm* revealed that gene flow between populations and between geographical regions was limited. The AMOVA analysis showed significant molecular divergences (84.7%) among geographical regions (mainland China and Taiwan), of which 10.2% could be attributed to variation among populations within regions, and only 5.2% to variation within populations (Table 4). For the mainland China populations, there was 44.77% molecular divergence among the four geographical regions in populations. These results implied that divergence in *C. ramondioides* populations was mainly caused by geographical isolation.

Recent migration rates between populations were estimated by BayesAss+, and included a statistical test calculating the posterior probability based on haplotypes of *GCYC1* (Table S5). Support for the existence of migrants was almost entirely absent in most populations. Significant recent introgression rates were found between all four of the populations in the G3 region from Lin'an. For all populations, estimates of *F* point to possible local inbreeding effects (Table S5, Table 3). Taken together, these results indicate that little gene flow exists between the sampled populations of *C. ramondioides*.

2.5 Mismatch distribution and assignment test

For detecting whether the sampled populations had undergone expansion, we calculated the mismatch

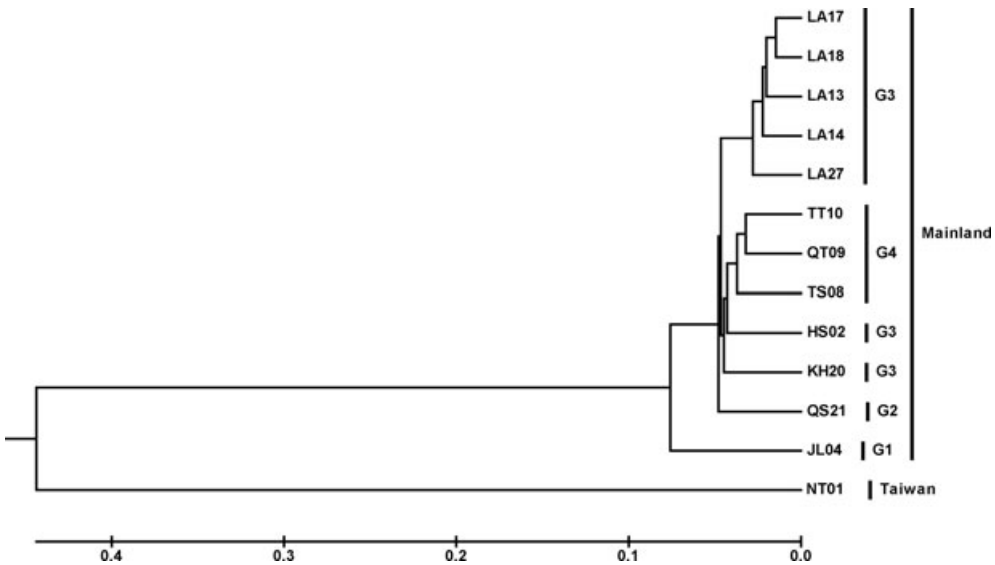

Fig. 4. UPGMA tree showing the relationships among the 13 sampled populations of *Conandron ramondioides* based on the genetic distance among *GCYC1* sequences. G1, Mount Wuyishan; G2, Mountains in western Anhui and Jiangxi provinces; G3, Mount Huangshan, mountains in western Zhejiang province and eastern Jiangxi province; G4, Low mountains and hills in eastern–southern Zhejing province (Tiantaishan–Kuocangshan–Donggongshan). HS02, Huangshan, Anhui; JL04, Longqishan, Jiangle, Fujian; KH20, Gutianshan, Kaihua, Zhejiang; LA13, Longtangshan, Lin'an, Zhejiang; LA14, Longtangshan, Lin'an, Zhejiang; LA17, Niulangping, Lin'an, Zhejiang; LA18, Dong'aotou, Lin'an, Zhejiang; LA27, Shunxi, Lin'an, Zhejiang; NT01, Fenghuangshan, Nantou, Taiwan QS21, Tianzhushan, Qianshan, Anhui; QT09, Kuocangshan, Qingtian, Zhejiang; TS08, Fenghuangshan, Taishun, Zhejiang; TT10, Tiantaishan, Tiantai, Zhejiang.

distribution of separate sites and analyzed pairwise differences and the Raggedness index (r) at the level of geographical region and of species by using DNASP (Rozas et al., 2003). This analysis could not be applied to the Taiwan population because of the limited number of individuals sampled (only five). Of the four geographical regions, three showed double or multiple peaks of mismatch distribution and no evidence was found from the Raggedness index for rejecting the stable population model. At the species level, the mismatch distribution with multiple peaks did not match expectations under the "sudden-expansion model" (Fig. S1). The Raggedness index and mismatch distribution suggested no demographic expansion across *C. ramondioides* populations.

2.6 Genetic divergence time between geographical regions

For further understanding of the correlation between geographic isolation and population genetic differentiation, we calculated the putative genetic divergence time between all five geographical regions according to the time of Taiwan island formation (Fig. 5). We assumed that Taiwan became an island approximately 5 Mya (Shen, 1994; Chiang & Schaal, 2006). The results showed that populations from geographical region G1 were first isolated from other regions approximately 841 000 years ago and populations from regions G2, G3, and G4 were formed from 545 000 to 512 000 years ago, in accord with Pleistocene glaciations, an epoch from 2.588 Mya to 11 700 years ago (Felix et al., 2004; Laurent et al., 2004).

3 Discussion

3.1 Population genetic diversity and phylogeographic pattern

Conandron ramondioides is characterized by high genetic differentiation among populations with an average $Fst = 0.814$ (Table 3). At the same time, there is little gene flow between local populations or closely related geographical regions, as indicated by Nm values that are almost zero ($Nm = 0.06$ or $Nm = 0.04$). These results are consistent with the populations being almost completely isolated from each other. As shown in the results, the homozygote outnumbers the heterozygote for all 36 sites in *GCYC1*. Such homozygote excess suggests that *C. ramondioides* has an appreciable selfing rate. Hand-pollination experiments on natural populations on *C. ramondioides* revealed that its flowers are self-compatible but not capable of auto-pollination (pers. obs. and pers. comm.). Thus, it is possible that an appreciable amount of insect-mediated self-pollination occurs in *C. ramondioides*, but detailed field observation on pollinators and their pollination behavior is needed. According to our

Table 3 Pairwise comparisons of *Fst* between populations and pairwise *Fst* F-statistics between geographical regions of *Conandron ramondioides*, based on haplotype frequencies of *GCYC1* ($P < 0.05$)

Population	NT01	TT10	TS08	QT09	QS21	LA13	LA14	LA17	LA18	LA27	KH20	JL04	HS02
NT01	—												
TT10	0.972	—											
TS08	0.905	0.298	—										
QT09	0.939	0.201	0.100	—									
QS21	0.931	0.518	0.357	0.354	—								
LA13	0.978	0.811	0.546	0.625	0.622	—							
LA14	0.977	0.812	0.545	0.630	0.627	0.024	—						
LA17	0.989	0.882	0.604	0.694	0.675	0.029	0.069	—					
LA18	0.984	0.844	0.566	0.647	0.642	0.018	0.087	0.093	—				
LA27	0.953	0.640	0.418	0.452	0.471	0.312	0.308	0.427	0.400	—			
KH20	0.952	0.448	0.209	0.156	0.338	0.618	0.625	0.708	0.650	0.400	—		
JL04	0.982	0.893	0.503	0.755	0.764	0.927	0.923	0.967	0.949	0.838	0.791	—	
HS02	0.978	0.763	0.474	0.518	0.580	0.861	0.860	0.925	0.892	0.702	0.536	0.919	—
Geographical region	TW	G1	G2	G3	G4								
TW	0												
G1	0.697	0											
G2	0.327	0.438	0										
G3	0.439	0.476	0.213	0									
G4	0.324	0.346	0.138	0.230	0								

G1, Mount Wuyishan; G2, Mountains in western Anhui and Jiangxi provinces; G3, Mount Huangshan, mountains in western Zhejiang province and eastern Jiangxi province; G4, Low mountains and hills in eastern–southern Zhejing province (Tiantaishan–Kuocangshan–Donggongshan); TW, Taiwan. HS02, Huangshan, Anhui; JL04, Longqishan, Jiangle, Fujian; KH20, Gutianshan, Kaihua, Zhejiang; LA13, Longtangshan, Lin'an, Zhejiang; LA14, Longtangshan, Lin'an, Zhejiang; LA17, Niulangping, Lin'an, Zhejiang; LA18, Dong'aotou, Lin'an, Zhejiang; LA27, Shunxi, Lin'an, Zhejiang; NT01, Fenghuangshan, Nantou, Taiwan QS21, Tianzhushan, Qianshan, Anhui; QT09, Kuocangshan, Qingtian, Zhejiang; TS08, Fenghuangshan, Taishun, Zhejiang; TT10, Tiantaishan, Tiantai, Zhejiang.

field observations, seeds are dispersed mainly by water streams after capsule dehiscing, limiting the long-distance dispersal of seeds, and reducing gene flow among populations. Furthermore, the populations had a relatively high level of haplotype diversity but a relatively low level of nucleotide diversity (Table S2). This implies a strong effect of population fragmentation, given that the nucleotide changes betray no evidence of selection.

Both the haplotype network and the NJ tree reveal two distinctive geographical groups, one in Taiwan and the other on mainland China (Figs. 2, 3). There are 20 nucleotide changes in the Taiwan population, of which 17 belong specifically to the Taiwan population (the remaining three are shared with Zhejiang populations in the mainland) (Table S2). That these two groups of populations are distinct is also strongly supported by AMOVA analysis of genetic differentiation (Table 4) and the genetic distance (Fig. 4). In addition, there are no common haplotypes between the Taiwan and mainland populations (Fig. 2 and Table S3). These results suggest a striking genetic differentiation between *C. ramondioides* populations in Taiwan and mainland China, although only one population and five individuals have been analyzed in Taiwan.

In mainland populations, there is evident genetic differentiation among the four geographical regions (Table 4). In addition, the frequencies of the central haplotypes Hap5 and Hap7 are many times higher than those of any other haplotype (Table S3). These two hub haplotypes are widespread in both Anhui and Zhejiang provinces, and more than half of the haplotypes are linked to Hap5 by one or two mutational steps (Fig. 2). According to Crandall & Templeton (1993), older haplotypes should be more broadly distributed geographically, and if this is true, then the haplotypes Hap5 and Hap7 are likely to be ancestral. Therefore, Anhui and Zhejiang provinces are suggestive of the original

Table 4 Analysis of molecular variance at different hierarchical levels for *Conandron ramondioides*, based on 496 sequences of 248 individuals using the *GCYC1* gene

Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation	*P*-value
Between mainland China and Taiwan	1	158.542	7.573	84.65	$P < 0.001$
Among populations within mainland China and Taiwan	11	408.253	0.908	10.15	$P < 0.001$
Within all populations of *C. ramondioides*	483	224.327	0.464	5.19	$P < 0.001$
Among four geographical regions in mainland China	3	258.196	0.744	44.77	$P < 0.001$
Among populations within mainland China	8	150.057	0.448	26.96	$P < 0.001$
Within all populations in mainland China	474	222.727	0.470	28.27	$P < 0.001$

d.f., Degrees of freedom.

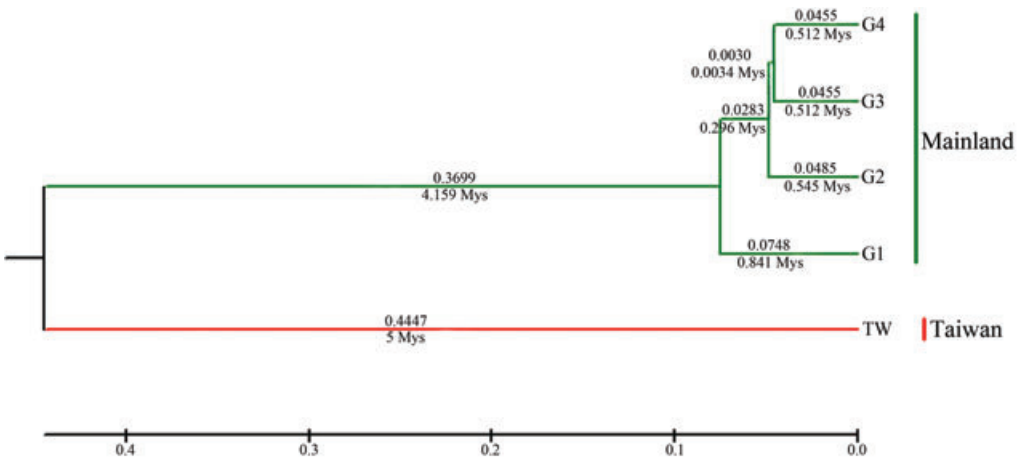

Fig. 5. UPGMA tree showing the evolutionary relationships between the five defined geographical regions based on the genetic distance among *Conandron ramondioides GCYC1* sequences. G1, Mount Wuyishan; G2, Mountains in western Anhui and Jiangxi provinces; G3, Mount Huangshan, mountains in western Zhejiang province and eastern Jiangxi province; G4, Low mountains and hills in eastern–southern Zhejing province (Tiantaishan–Kuocangshan–Donggongshan).

population range of this species. Similarly, populations with the highest *GCYC1* nucleotide and haplotype diversities are found in these two provinces.

The phylogeographic pattern of a plant species is affected not only by biological and ecological traits but also evolutionary history. Prior to the first cold period of the Quaternary, central and southeastern China (i.e., present day Zhejiang and Anhui) was continuously covered by tropical or subtropical humid forests (Wu & Wang, 1983). As the climate became colder and drier during the Pleistocene, species adapted to humid habitats disappeared from the main part of these areas, and became restricted to relict populations, where the habitat remained suitable, scattered within the original distribution. This pattern is exemplified by the so-called living fossils, e.g., *Metasequoia glyptostroboides* Hu & Cheng., *Ginkgo biloba* L., *Davidia involucrata* Baill., and *Emmenopterys henryi* Oliv. (Wu & Wang, 1983; Zhang, 2004). This pattern can be predicted to apply to the species *C. ramondioides*, which is adapted to the shady and moist habitat under humid-temperate forests (Xiao & Wang, 2007). A continuous distribution in central, eastern, and southeastern China could have fragmented during the Pleistocene into small and isolated populations over this range. The regions of Anhui–Jiangxi (central China) and Zhejiang (eastern China) are likely to be the refugial areas of this species.

In addition, in the mainland haplotypes exclusive to a single population, seven of them belong to the Qianshan (QS21) population, four to the Huangshan (HS02) population, and three to the Kaihua (KH20) population, which all are in central China. The other 14 unique haplotypes are spread out among various populations. The haplotype richness found in central China, along with the two network hub haplotypes occurring predominantly in Anhui and Jiangxi, suggest that the central China populations might be close to the ancestral *C. ramondioides*.

3.2 Correlation between population genetic differentiation and geographic isolation at different timescales

The island of Taiwan, facing the Pacific Ocean in the east, is separated from the mainland by the Taiwan Strait. However, prior to the late Cretaceous, the island was part of a section of the Andes-type continental margin along the eastern edge of the mainland (Shen, 1994). During the late Cretaceous or Eocene, this region underwent intense back-arc expansion to create a series of rift basins, trending roughly northeast–southwest, that were then filled with regional subsidence, tilting the continental margin seaward (Shen, 1994; Chang et al., 2001; Yu & Chou, 2001). The geotectonic processes along the eastern edge of China are characteristic of a rifting continental margin with continuous regional subsidence. Beginning in the Late Miocene, approximately 5–6 Mya, this region underwent a strong uplift consequent to the oblique collision between the Eurasian plate and the Philippine Sea plate. This collision led to active volcanic eruptions and to the emergence of the Taiwan Strait.

Correlatively, the island of Taiwan not only shows a high similarity to the central–eastern mainland in extant flora but also has numerous living or fossil plants that were prominent in the tertiary humid-temperate forests

of the mainland (Shen, 1994; Ying & Hsu, 2002). As a possible component of the tertiary humid-temperate forest understory, *C. ramondioides* might have been widely distributed in Taiwan and central-east China during the Miocene. If so, then the isolation of the present-day populations between Taiwan and the mainland might have begun approximately 5 Mya. In contrast, disruption to the mainland populations would have occurred much later, possibly not until the glaciation of the Pleistocene, which resulted in considerable population reduction and habitat fragmentation of numerous species of the tertiary humid-temperate forest in central and eastern mainland China, as well as Taiwan Island (Wu & Wang, 1983; Shen, 1994; Gong et al., 2008; Yu & Chou, 2001; Wang et al., 2004b; Su et al., 2005).

In addition to island formation and glaciations, the present-day phylogeographic pattern of *C. ramondioides* might have been shaped to a significant extent by human activities since Neolithic times (approximately 10 000–2000 years BC, the last part of the Stone Age covers the period from the onset of farming and ending when metal tools came into widespread use), with large tracts of natural forest continuously displaced by agricultural areas, especially in the low altitude areas in central and eastern mainland China (Wu & Wang, 1983). For example, populations in northwestern Jiangxi (central China) collected 50 years ago in Xiushui and Jing'an are apparently extinct, presumably as a result of deforestation and crop planting, an impact of modern agricultural activities. These results indicate that the habitat of *C. ramondioides* has fragmented in successive eras, consequent upon island formation, glacial impacts, and human activities.

Not surprisingly, the extent of population genetic differentiation among the sampled regions is positively correlated with successive habitat fragmentation. Based on the assumed time for the formation of Taiwan, we calculated the putative genetic divergence time between geographical regions based on distance data (Fig. 5). The results strengthen the correlation between the population genetic differentiation and geographic isolation from both island formation and glaciation. According to the results, the population genetic divergences among the mainland regions range from 512 000 to 841 000 years ago, times that are consistent with fluctuating climate during the glacial period.

3.3 Molecular evolution of *GCYC1*

In the model species *Antirrhinum majus* L., *CYC* genes are essential for the development of dorsoventral asymmetry in flowers. The gene specifies a dorsal fate for floral organs conditioning the production of zygomorphic flowers with distinct dorsal and ventral structures. Consistently, the loss-of-function of *CYC* gives rise to actinomorphic mutants with five equal petals and five (or six) fertile stamens (Luo et al., 1996, 1999). In Lamiales, a major clade of predominantly zygomorphically flowered angiosperms, the zygomorphy groups with bilabiate corolla and four fertile stamens plus a staminode are believed to be ancestral to actinomorphy (Donoghue et al., 1998; Endress, 1998, 1999; Cubas, 2004; Zhou et al., 2008). The derived actinomorphic flowers are associated with *CYC*-like gene silencing or down-regulation, as in *Linaria* (Veronicaceae), *Sinningia,* and *Bournea* (Gesneriaceae), members of two families at the basal position in Lamiales (Cubas et al., 1999a, 1999b; Citerne et al., 2003; Cubas, 2004; Zhou et al., 2008).

The species *C. ramondioides*, as a representative of a derived actinomorphic lineage in Gesneriaceae, shows no residue of zygomorphy (Wang et al., 2010). Among the sampled populations, we found no evident floral morphological variation. Although not tested directly, the above facts imply that, in *C. ramondioides, GCYC* might be silenced or strongly down-regulated. This assumption is supported by the analysis of the *GCYC1* nucleotide sequences which gave no evidence to reject the hypothesis of neutral evolution at this locus (Table 2). Therefore, the strikingly different patterns of nucleotide and haplotype divergence in the mainland and Taiwan populations, as well as the high level of substitution polymorphism among different geographic regions within the mainland, testify to the evolutionary history of the various sampled populations of *C. ramondioides*.

Acknowledgements The authors thank Professor Song GE (Institute of Botany, Chinese Academy of Sciences, Beijing, China) and Professor Tobias BASKIN (Biology Department, University of Massachusetts, Amherst, MA, USA) for advice aiding the development and refining of the English language of this manuscript. We also thank Dr. Ping WAN (College of Life Science, Capital Normal University, Beijing, China) and Dr. Xiaoming ZHENG (State Key Laboratory of Systematic and Evolution Botany, Institute of Botany, Chinese Academy of Sciences, Beijing) for help in data analysis. We are grateful to Professor Changfu XIE (Department of Life Science, National Taiwan University, Taiwan) for help in collecting plant materials in Taiwan.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1 Mismatch distribution across populations of *Conandron ramondioides*, showing the observed pairwise nucleotide site differences (dotted line) and the expected (solid line) obtained with DNASP.

Table S1 Genetic distance Kxy between 13 populations of *Conandron ramondioides*

Table S2 Variable sites of the aligned sequences of *GCYC1* sequences in 41 haplotypes of *Conandron ramondioides*

Table S3 *GCYC1* gene haplotype distribution and frequencies in 13 populations of *Conandron ramondioides*

Table S4 Summary of statistics for intragenic recombination at the *GCYC1* locus in all sampled populations, geographical regions, and species. *C*, estimator of the population's recombination rate per site, as given by Hudson (1987); *Rm*, minimum number of recombination events, as given by Hudson & Kaplan (1985)

Table S5 Means of the posterior distribution of the migration rates (*m*), and means and standard deviations of the posterior distribution of the inbreeding coefficient (F) of each population estimated using the program BayesAss+

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