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Refugia and phylogeography of *Taiwania* in East Asia

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ABSTRACT

Aim The East Asia endemic *Taiwania cryptomerioides* Hayata is an iconic and relictual monotypic conifer whose main extant populations are now restricted to the Yunnan–Myanmar border, northern Vietnam and Taiwan. It has also been reported from several localities in Guizhou, Hubei and Fujian Provinces, China. Its fossil record indicates that, while it was more widely distributed in the Northern Hemisphere and grew under a range of different ecological conditions, it has remained almost unchanged in its morphology for over 100 Myr. We investigate whether these remaining extant, disjunct populations have diverged genetically; when such a divergence may have occurred; and which, if any, of the extant populations exhibit refugial characteristics.

Location East Asia.

Methods Sequences of five chloroplast DNA markers (*petG–trnP*, *trnH–psbA*, *trnV–trnM*, *trnC–ycf6* and *trnL–trnF*) from all extant populations of *T. cryptomerioides* were analysed to reveal their phylogeography. Molecular clock models with fossil calibrations were used to estimate divergence times between extant populations.

Results Extremely low nucleotide diversity was found in the overall population (π = 0.00077) with only nine haplotypes distinguished. The mainland Asia populations share one major ancestral haplotype. The insular populations in Taiwan all possess a unique haplotype with at least an eight-mutational-step difference to the mainland Asia haplotype. Molecular clock estimations demonstrated that the mean divergence time between the predominant insular population haplotype and the mainland Asia haplotype occurred at *c.* 3.23–3.41 Ma, followed by a split into Vietnamese and Yunnan–Myanmar populations (*c.* 1.0–1.39 Ma).

Main conclusions Strong genetic differentiation exists between insular (Taiwan) and mainland Asia populations. The split between insular and mainland haplotypes can be dated back to the end of the Pliocene. The Yunnan–Myanmar border area, northern Vietnam and Taiwan are identified here as potential refugia for *T. cryptomerioides*. Other populations in mainland China are unlikely to be the result of historical fragmentation and their origins require further investigation.

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INTRODUCTION

During the Pleistocene, many plant taxa across the Northern Hemisphere survived in areas that acted as refugia (Comes & Kadereit, 1998; Hewitt, 1999, 2004; Petit *et al.*, 2003). The locations of these refugia and post-glacial recolonization routes for some plant species have been well studied in Europe and northern America (Tiffney & Manchester, 2001; Petit *et al.*, 2003; Milne, 2006; Médail & Diadema, 2009). However, much less is known about Asian refugia and potential recolonization routes in Asia (Hewitt, 2000, 2001, 2004).

Southern China, southern Japan and Taiwan are noted for their high levels of plant endemism and rich floral diversity (Axelrod *et al.*, 1998; Aoki *et al.*, 2004; Cheng *et al.*, 2005; Chiang *et al.*, 2006). One explanation for these high levels is that during the Pleistocene the climate in these areas was less severe than in other parts of the Northern Hemisphere, where it was cold and dry and glaciation was extensive (Shi *et al.*, 1999; Shen, 2002; Zhao *et al.*, 2003; Ehlers & Gibbard, 2007). This, combined with a complex, microhabitat-rich topography, allowed many plant taxa to survive. Such areas have been proposed as potential glacial refugia (Shen *et al.*, 2005; Wu *et al.*, 2006; Manchester *et al.*, 2009).

These parts of eastern Asia are characterized by the presence of a number of iconic, relictual monotypic or almost monotypic gymnosperm genera that were once widespread across the Northern Hemisphere. Examples include *Cathaya*, *Cryptomeria*, *Cunninghamia*, *Ginkgo*, *Glyptostrobus*, *Metasequoia*, *Pseudolarix* and *Taiwania*. As climate deteriorated globally during the Late Miocene–Pliocene, the geographical extent of their former widespread distribution contracted. In many cases, their last known macrofossil occurrence is recorded in Pliocene- and Pleistocene-age sediments in Japan (Miki, 1954; LePage & Basinger, 1995; LePage *et al.*, 2005; Momohara, 2005; LePage, 2007, 2009). With the exception of *Cryptomeria*, all the above-mentioned taxa grow in southern China, Taiwan and Indochina, where they either have restricted, localized populations (*Cathaya*, *Metasequoia* and *Pseudolarix*) or have wider distributions that reflect their long history of human cultivation (*Ginkgo*, *Cryptomeria*, *Cunninghamia* and *Glyptostrobus*). *Taiwania* is perhaps an exception to both, as its major remaining populations are geographically widespread across East Asia and it has never been widely cultivated.

The main extant populations of *Taiwania* are restricted to Taiwan, northern Vietnam, and the border area between Yunnan and Myanmar (Gaoligongshan). In these areas, it occurs on steep slopes in moist, evergreen forests at elevations ranging from 1800 to 2500 m (Fig. 1a; Farjon & Thomas, 2007; Li *et al.*, 2008b). Annual precipitation may be as high as 4000 mm, while the mean annual temperature varies from 9 to 15 °C (Liu & Su, 1983; Hiep *et al.*, 2004; Lai *et al.*, 2006; Li *et al.*, 2008a). Along the Yunnan–Myanmar border, it is associated with *Tsuga dumosa* and various members of the Fagaceae, Lauraceae, Theaceae and Araliaceae. In the past, these western populations were treated as distinct species

(*Taiwania flousiana* Gaussen and *Taiwania yunnanensis* Koidzumi) on the basis of perceived differences in cone size and the number of seed cone scales (Cheng & Fu, 1978). More recent taxonomic revisions have found that these variations are common across the entire range of the genus and therefore such works recognize only a single species: *Taiwania cryptomerioides* Hayata (Wu & Raven, 1999; Farjon, 2005).

In Vietnam, *Taiwania* is known from one location in the Hoang Lien Mountains, an area that represents the most southern extension of the Himalayas. This area shares many climatic and floristic similarities with the areas of the Yunnan–Myanmar border, where *Taiwania* also occurs, although in Vietnam it is associated with *Fokienia hodginsii* (Farjon & Thomas, 2007) rather than *Tsuga*. The single known stand is a small fragment of a larger population that has recently been reduced by shifting cultivation (Hiep *et al.*, 2004).

In Taiwan, the species also occurs in evergreen forests, but with a much greater diversity of conifers, including *Chamaecyparis obtusa* var. *formosana*, *Chamaecyparis formosensis* and *Cunninghamia konishii*. Its current sporadic distribution along the main north–south mountain range is the result of exploitation over the past century: in the recent past it was more frequent and more continuously distributed.

Taiwania may reach a height of 80 m and an age of up to 1600 years (Hu, 1950). Its timber is highly valued and has been heavily exploited throughout its range. Currently, it is assessed as globally vulnerable by the IUCN, with regional assessments of either critically endangered in Vietnam (Nguyen, 2007), or endangered in Taiwan and Yunnan (Wang & Xie, 2004; Farjon & Thomas, 2007).

Taiwania is also known to occur in several localities in Guizhou, Hubei and Fujian Provinces of China (Fig. 1a). In these areas, populations of fewer than 100 mature trees are found scattered in secondary forests at lower elevations (750–1200 m). Precipitation ranges from 500 to 1200 mm (Liu & Su, 1983; Fu & Jin, 1992; Li & Li, 2005). The contrast in their ecological profiles compared with those of the main populations, as well as their proximity to human settlements, has led to some debate about whether they are the remnants of a more extensive and contiguous population, the result of recent natural recolonization/recruitment, or the result of human introduction (Yang *et al.*, 2006, 2009; Farjon & Thomas, 2007; Li *et al.*, 2008b).

Historical migration, fragmentation, colonization and refugium locations can be inferred by phylogeographical studies (Avise, 2000). Chloroplast DNA (cpDNA) is the most optimal marker for phylogeographical studies in plants because of its low recombination rate, small effective population size (Comes & Kadereit, 1998) and low mutation rate (Zurawski *et al.*, 1984; Wolfe *et al.*, 1987; Zurawski & Clegg, 1987). Analyses of genetic diversity in cpDNA markers have indicated the possible existence of refugia in large continents such as Europe (Ferris *et al.*, 1993) or North America (Soltis *et al.*, 1997), and in small geographical regions such as Taiwan (Cheng *et al.*, 2005; Wu *et al.*, 2006).
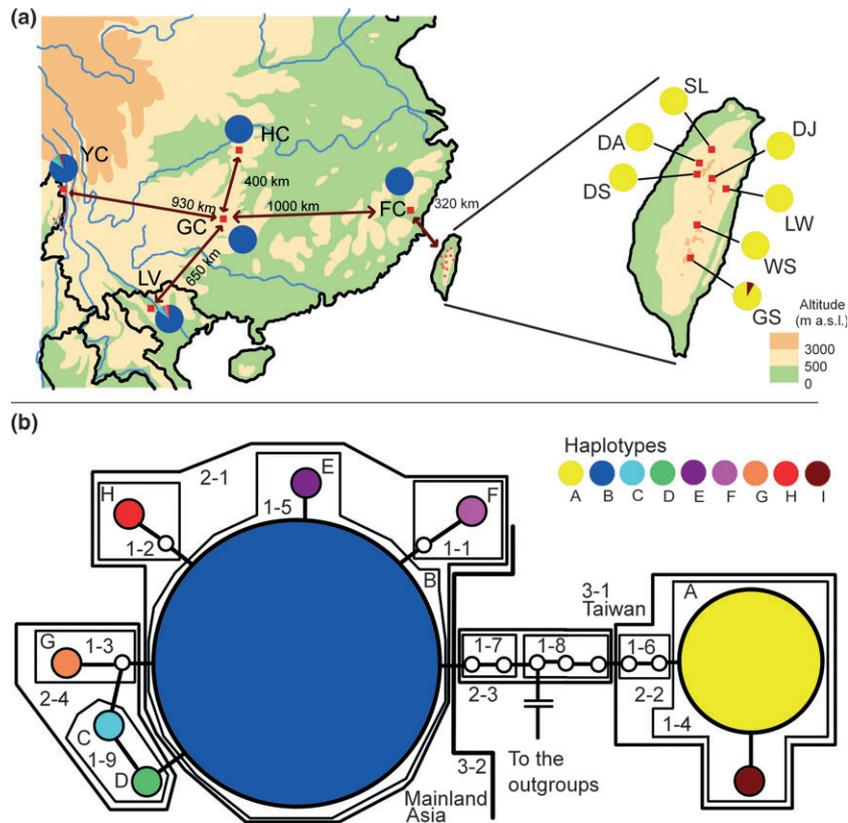
Figure 1 Geographical distributions of cpDNA haplotypes of *Taiwania* and the haplotype network. (a) Distribution of the eight cpDNA haplotypes of *Taiwania* from the extant populations in Taiwan, China and Vietnam. Sampling locations are marked with red squares. Population codes are abbreviated following the labels used in Table 1. Distances between major populations are indicated by dark red arrows. (b) Statistical parsimony network depicting the cpDNA haplotype relationships of *Taiwania*. Each line between haplotypes indicates a mutational step. A small, open circle represents a hypothetical haplotype with another mutational step between real haplotypes. Between haplotypes A and B, the deletion–insertion gap at 309–316 bp of *pet*G–*trn*P listed in Table 1 is treated as a single mutation step. The haplotypes/clades belonging to the same nested clade are grouped within polygons. The connection between the network and outgroup is simplified because of the large number of mutations (400 steps). The size of each circle is proportional to the haplotype frequency.

The evolutionary history of cpDNA haplotypes can be revealed by statistical methods such as the nested clade phylogeographical analysis (NCPA; Templeton, 1998, 2004). Moreover, molecular dating has been applied in recent phylogeographical studies of other East Asian conifers to estimate times of haplotype lineage divergences (*Chamaecyparis*: Wang *et al.*, 2003; *Pinus luchuensis*: Chiang *et al.*, 2006; *Taxus*: Gao *et al.*, 2007).

In this study, five cpDNA markers (*pet*G–*trn*P, *trn*H–*psb*A, *trn*V–*trn*M, *trn*C–*ycf*6 and *trn*L–*trn*F) were used to infer the phylogeographical relationship of extant *Taiwania* populations. These markers have recently been successfully applied in a range of other modern phylogeographical studies (*Cunninghamia*: Hwang *et al.*, 2003; *Castanopsis carlesii*: Cheng *et al.*, 2005; *Quercus mongolica*: Okaura *et al.*, 2007). Using cpDNA phylogeographical analysis, we sought to investigate the following: (1) whether the disjunct *Taiwania* populations have diverged genetically; (2) when such a divergence might have occurred; and (3) which, if any, extant populations exhibit refugial characteristics.

MATERIALS AND METHODS

Sample collection

Samples from a total of 153 individuals of *Taiwania* were collected from Taiwan, China, and Vietnam (Table 1; Fig. 1a). Leaves from 55 trees were collected from seven geographically distinct populations around Taiwan, growing in the conservation seed orchards at the Lienhuachih (Nantou County) and Liukuei Stations (Kaohsiung County), Taiwan Forestry Research Institute (TFRI). Each sample from the seed orchard represented a single progeny raised from seed collected from one seed tree within a particular area/provenance. No siblings were sampled. Sixty-four samples from trees growing in southern China (Guizhou, Hubei and Fujian Provinces) and the Yunnan–Myanmar border were collected. Thirty-four samples were collected from the population in Lao Cai Province, Vietnam.

Given that *Cunninghamia* is reported to be the genus most closely related to *Taiwania* based on morphology (Farjon &

Table 1 Locations of the *Taiwania* populations studied: estimates of nucleotide diversity (π) and haplotype diversity (*h*) in each population are indicated, together with values of the neutrality tests (Tajima's *D*, Fu and Li's *D** and *F**) for each geographical region.

Acronym	Locality	Region	Coordinates (N/E)	Elevation range (m a.s.l.)	Haplotype (number of individuals)	π	h	Tajima's D	Fu and Li's D*	Fu and Li's F*
SL	Siouluan, Hsinchu County	Taiwan	24°37'/121°15'	c. 1800	A (7)	0.00000	0.000	N/A	N/A	N/A
DA	Daansi, Taichung County	Taiwan	24°26'/121°04'	1800–2000	A (4)	0.00000	0.000	N/A	N/A	N/A
DJ	Dajian, Taichung County	Taiwan	24°14'/121°15'	1900–2200	A (8)	0.00000	0.000	N/A	N/A	N/A
DS	Dasyueshan, Taichung County	Taiwan	24°17'/121°02'	1800–2200	A (10)	0.00000	0.000	N/A	N/A	N/A
WS	Wangsiang, Nantou County	Taiwan	23°37'/121°02'	1800–2600	A (10)	0.00000	0.000	N/A	N/A	N/A
LW	Liwusi, Hualien County	Taiwan	24°05'/121°27'	1800–2300	A (4)	0.00000	0.000	N/A	N/A	N/A
GS	Guanshan, Taitung County	Taiwan	23°11'/120°56'	2000–2300	A (11), I (1)	0.00006	0.167	−1.14053	−1.32974	−1.44334
YC	Around Qi Qi Nature Reserve Station, Gongshan County, Yunnan Province, near Myanmar border	China	27°37'–27°43'/98°35'–98°40'	1900–2200	B (16), C (1), D (1), E (1)	0.00015	0.298	−1.42238	−1.22514	−1.46876
GC	Leishan county, Guizhou Province	China	26°34'/108°15'	1040	B (15)	0.00000	0.000	N/A	N/A	N/A
HC	Lichuan county, Hubei Province	China	30°04'/109°05'	850	B (15)	0.00000	0.000	N/A	N/A	N/A
FC	Gutian and Pingnan Counties, Fujian Province	China	26°35'–26°56'/118°48'–119°11'	400–880	B (15)	0.00000	0.000	N/A	N/A	N/A
LV	Liem Phu Commune, Van Ban District, Lao Cai Province	Vietnam	21°56'/104°19'	1800–2100	B (29), C (2), F (1), G (1), H (1)	0.00017	0.275	−1.65737	−1.56337	−1.85395
		Total			(153)	0.00077	0.532	0.07443	−2.14384	−1.59976

N/A, not applicable.

Garcia, 2003) or a combination of morphological and molecular characters (Gadek *et al.*, 2000), we selected *C. konishii* as the outgroup for construction of the haplotype network and divergence time estimations.

DNA extraction, polymerase chain reaction and sequencing

Genomic DNA was extracted using the modified cetyltrimethyl ammonium bromide (CTAB) method (Doyle & Doyle, 1987). All of the extracted DNA was dissolved in 40 μL double-distilled water and diluted to a concentration of *c.* 20 ng μL^{-1} for the PCR reactions.

PCR was performed to amplify five cpDNA regions: *pet*G–*trn*P (5′-GGTCTAATTCCTATAACTTTGGC-3′ and 5′-GGGATGTGGCGCAGCTTGG-3′, Hwang *et al.*, 2000); *trn*V–*trn*M (5′-GCTATACGGGCTCGAACC-3′ and 5′-TACCTACTATT GGATTTGAACC-3′, Hwang *et al.*, 2000); *trn*H–*psb*A (5′-CGCGCATGGTGGATTCACAATCC-3′ and 5′-GTTATGCAT GAACGTAATGCTC-3′, Tate, 2002; Tate & Simpson, 2003); *trn*C–*ycf*6 (5′-CCAGTTCRAATCYGGGTG-3′ and 5′-GCCC AAGCRAGACTTACTATATCCAT-3′, Shaw *et al.*, 2005); and *trn*L–*trn*F (5′-CGAAATCGGTAGACGCTACG-3′ and 5′-ATT TGAACTGGTGACACGAG-3′, Taberlet *et al.*, 1991). The PCR reactions were performed using the *Prime Taq* DNA polymerase (GENET BIO, Nonsan, Korea). The PCR profile was programmed with an initial denaturation of 3 min at 94 °C, followed by 35 cycles of 45 s at 94 °C, 45 s at 55 °C and 1 min 30 s at 72 °C, with a final extension for 7 min at 72 °C. The purified products were sequenced by ABI 3730 DNA Analyzer (Applied Biosystems, Foster City, CA, USA) using BigDye Terminator v. 3.1 (Applied Biosystems). Sequences obtained were deposited in GenBank under accession numbers JF514856–JF514876.

Data analyses

The sequence data for all individuals were aligned by hand using BioEdit v. 7.0.5.2 (Hall, 1999). Results of neutrality tests including Tajima's *D* and Fu & Li's *D** and *F** (Tajima, 1989; Fu & Li, 1993) indicated that all five cpDNA regions are selectively neutral (Table 1). The result of the partition homogeneity test (incongruence length difference test; Farris *et al.*, 1994) revealed no character incongruence (*P* = 1) among all five cpDNA regions. On that basis, we combined the five regions and treated them as a single locus in our subsequent analyses.

Measures of genetic variations, such as haplotype diversity (*h*) and nucleotide diversity (π), were calculated using DnaSP v. 4.50.0.3 (Rozas *et al.*, 2003). To estimate the extent of genetic divergence and gene flow among the geographical regions (Taiwan, China and Vietnam), measures of pairwise genetic differentiation (F_{ST}) and gene flow (*Nm*) among geographical regions were calculated using Arlequin v. 3.11 (Excoffier *et al.*, 2005). To quantify further the genetic differentiation partitioned among different geographical regions

(China, Vietnam and Taiwan); between populations in Taiwan and mainland Asia (China and Vietnam); and between populations in each of those regions, analysis of molecular variance (AMOVA) (Excoffier *et al.*, 1992) in Arlequin was applied. The Kimura two-parameter model was chosen because the amount of variation was small, and significance of the variance components at each hierarchical level was tested with 1000 random permutations.

The phylogeographical relationships between *Taiwania* haplotypes were elucidated by a statistical parsimonious haplotype network rooted on *C. konishii* using TCS v. 1.21 (Clement *et al.*, 2000). The statistical support limit was set at 0.95 for connections between *Taiwania* haplotypes and at 400 mutational steps for connections between outgroup and *Taiwania* haplotypes. Gaps were treated as the fifth character state regardless of the length. The haplotype network was nested according to Templeton & Sing's (1993) nesting rule. To deduce the evolutionary history that could have shaped the observed geographical distribution of the recorded haplotypes among *Taiwania* populations, the nested network was applied to the NCPA (Templeton, 1998) using GeoDis v. 2.5 (Posada *et al.*, 2000). The clade distance, *Dc*, measures the spatial spread of haplotypes within each clade; the nested clade distance, *Dn*, calculates the spatial spread of each haplotype or a clade to a higher-level clade centre where it is nested. The outcome for its evolutionary history was inferred from the modified inference key released by the authors on 11 November 2005.

Estimate of divergence times

We chose two fossils to calibrate the molecular clock and establish divergence times. The first calibration point marks the common ancestor of both *Taiwania* and *Cunninghamia* from the earliest known occurrence of Cupressaceae assignable to a modern genus (Krassilov, 1967; Meng *et al.*, 1988; Yu, 1995), around the Early Cretaceous (Hauterivian: *c.* 145.5 to 136.4 Ma). The second calibration point was for the most recent common ancestor of extant *Taiwania* populations and was derived from the most recent recognizable fossil occurrence of modern *Taiwania* from the Pliocene in Japan (*c.* 5.3–2.58 Ma) (Miki, 1954; International Commission on Stratigraphy, 2008; LePage, 2009).

The number of nucleotide substitution was calculated directly from our combined cpDNA data using Kimura's three substitution types model, which assumes different rates for two kinds of transversions to correct the bias in favour of transition events (Kimura, 1981; Zurawski *et al.*, 1984). After dividing the number of substitutions per site by the time range of the most recent fossil record of *Taiwania* in the Pliocene (*c.* 5.3–2.58 Ma), the average substitution rate μ was normalized and set the mean at 1.40×10^{-9} mutations per site per year with standard deviation equals to 2.3×10^{-10}. We use this substitution rate for all the following analysis priors. We aim to calculate the divergence time of populations from major geographical regions: (1)

between Taiwan and mainland Asia, and (2) within mainland Asia.

Bayesian statistical methods offer the opportunity of exploring a wide diversity of alternative models with different assumptions on the shape of the tree and changes in the substitution rate over time (LePage *et al.*, 2007; Forest, 2009). We chose three specific Bayesian parametric approaches, considering either species tree topology or gene (haplotype) tree topology, or without any tree topology, for estimations of taxa divergence times. Two independent analyses were performed for each method to check the consistency of the results.

First, we used the MCMCcoal (Rannala & Yang, 2003) software package v. 1.2 to estimate divergence times on a known species tree based on a strict molecular clock with one global rate of substitution. We constrained the species tree-setting individuals from Taiwan and those from mainland Asia as two species. The parameters of divergence time τ for each internal node on the given species tree were estimated using the equation $\tau = \mu t$, in which μ is the average substitution rate 1.40×10^{-9} from our combined cpDNA data and t (time) is normalized with the two fossil calibration times mentioned above. The alignment gaps were treated as missing data. Each independent analysis was run for 200,000 generations with trees sampled every two generations and the first 10,000 burn-in phase trees discarded.

Second, we selected an approach to estimate divergence times depending upon the overall *Taiwania* haplotype tree topology. The PAML/MULTIDIVTIME software, which combines PAML v. 4 (Yang, 2007) and MULTIDISTRIBUTE v. 9/25/03, was used (Thorne *et al.*, 1998; Kishino *et al.*, 2001) following the guidance published by Rutschmann (2005). This approach allows the substitution rate to change along lineages with rate autocorrelation for divergence time estimation. The phylogenetic relationships among haplotypes were reconstructed using PAUP* v. 4.0b10 (Swofford, 2003) to obtain maximum parsimonious (MP) trees. The heuristic search algorithm was applied with 10,000 replicates of RANDOMADDITIONSEQUENCE, followed by tree bisection–reconnection (TBR) branch swapping on all the resulting trees with MULTREES, STEEPEST DESCENT, branch COLLAPSE (MAXBRLEN) on and ACCTRAN optimization to increase the chance of including all islands of most parsimonious trees (Wang *et al.*, 2004). As the PAML/MULTIDIVTIME software suggests using the best tree topology without saving branch lengths for estimation prior, the strict consensus tree from the resulting 35 MP trees was chosen for the input tree that was topologically similar to our haplotype network. Estimates of the transition/transversion rate ratio and the gamma site class-specific rates were calculated by PAML v. 4b (Yang, 2007) under the F84 + G model. Output data of PAML were used to calculate the maximum likelihood of the branch lengths and estimate divergence times by the programs PAML2MODELINF, ESTBRANCHES_DNA and MULTIDIVTIME in the package MULTIDISTRIBUTE (Thorne *et al.*, 1998; Kishino *et al.*, 2001). The established branch length, normalized substitution rate and the fossil calibration time in the Pliocene (maximum 5.3 and minimum 2.58 Ma) were set as the priors for MULTIDIVTIME to estimate posterior divergence times with a Markov chain Monte Carlo (MCMC) procedure. The MCMC was run for 100,000,000 generations with trees sampled every 1000 generations and the first 1,000,000 burn-in phase trees discarded.

The third approach estimates divergence times with the software BEAST v. 1.4.8 (Drummond & Rambaut, 2007). The time estimation in BEAST does not depend on a single tree topology as a prior, but is directly inferred by the sequence data and uncorrected rates are assumed. The best-fit substitution model selected for our sequence data was HKY85 according to Bayesian information criterion implemented through jMODEL TEST (Posada, 2008). We used a fixed substitution rate 1.40×10^{-9} that was estimated from our data. The Yule tree prior was chosen as it assumes a constant speciation rate in stochastic lineage splitting process for closely related species. Two *Taiwania* fossil calibration times, converted into normal distributions (the earliest common ancestor of both *Taiwania* and *Cunninghamia* is set at the Early Cretaceous: 122.5 ± 7.65 Ma and the most recent common ancestor of all living *Taiwania* is set at the Pliocene: 3.569 ± 1.763), were also incorporated as the priors because we assumed that the ages of these two nodes are equally likely to be older or younger than the actual age of the fossil. For the analysis, the MCMC was run for 100,000,000 steps each chain and sampled every 1000 steps after the first 1,000,000 steps of each run were discarded as burn-in. The posterior distributions of the divergence times (time to most recent common ancestor, TMRCA) of *Taiwania* from major geographical regions were generated using TRACER v. 1.4 (Rambaut & Drummond, 2007).

RESULTS

Genetic diversity and differentiation of *Taiwania* populations

The five cpDNA regions amplified in this study have a combined length of 2651 bp, in which three, *petG*–*trn*P (433 bp), *trn*H–*psb*A (534 bp) and *trn*L–*trn*F (748 bp), showed polymorphisms. Seventeen polymorphic sites were found, including 11 substitutions and six indels (Table 2). The *trn*V–*trn*M (681 bp) and *trn*C–*ycf*6 (255 bp) regions showed no polymorphisms.

Nine haplotypes were found in populations of *Taiwania* across its distributional range (Tables 1 & 2). Haplotypes A and I are unique to Taiwan and were the only haplotypes found in Taiwan. Haplotype I was restricted to a single individual in population GS from south-eastern Taiwan. On the mainland, haplotype B was common to all populations and was the only haplotype present in the Fujian, Hubei and Guizhou populations. Haplotype C was common to the Yunnan and Vietnam populations. Three haplotypes (F, G and H) were unique to Vietnam, while two others (D and E) were unique to Yunnan (Table 1; Fig. 1a). The Vietnamese population shows the highest nucleotide diversity ($\pi = 0.00017$), while the Yunnan population showed the

Table 2 Nucleotide polymorphic sites in nine cpDNA haplotypes of *Taiwania*.

Haplotype	Number of alleles	*pet*G–*trn*P (433 bp)						*trn*H–*psb*A (534 bp)						*trn*L–*trn*F (748 bp)				
		113	114	254	291	309–316	363	25	31	114	158	223	385	69	517	706	714	738
A	42(Tw)	G	G	T	G	–	G	A	T	G	C	A	–	C	–	G	–	G
B	61/29 (Cn/Vn)	.	.	C	.	#	.	.	.	–	C	T	G	A	T	.	–	.
C	1/2 (Cn/Vn)	.	.	C	A	#	A	.	.	–	C	T	G	A	T	.	–	.
D	1 (Cn)	.	.	C	A	#	.	.	.	–	C	T	G	A	T	.	–	.
E	1 (Cn)	.	.	C	.	#	.	.	T	–	C	T	G	A	T	.	–	.
F	1 (Vn)	C	A	C	.	#	.	.	.	–	C	T	G	A	T	.	–	.
G	1 (Vn)	.	.	C	.	#	.	A	.	–	C	T	G	A	T	–	–	.
H	1 (Vn)	.	.	C	.	#	.	.	.	–	C	T	G	A	T	.	C	A
I	1 (Tw)	.	.	.	.	–	.	.	.	.	T	.	.	.	–	.	–	.

# indicates a major insertion site (TGATAAAT) which separates other haplotypes (mainland Asia populations) from haplotype A (all Taiwanese populations). Dot (.) indicates sites sharing the same states as haplotype A; – indicates an alignment gap. Tw, Taiwan; Cn, China; Vn, Vietnam.

Table 3 Pairwise genetic differentiation (F_{ST}, above diagonal) and gene flow (Nm, below diagonal) estimates between geographical regions of *Taiwania* populations based on genetic variation of cpDNA.

	Taiwan	China	Vietnam
Taiwan	–	0.97953***	0.95346***
China	0.01045	–	0.01449
Vietnam	0.02441	33.99915	–

Levels of significance are based on 1000 random permutations in which $P < 0.001$ is marked by ***.

highest haplotype diversity ($h = 0.275$). In contrast, the populations in Guizhou, Hubei, Fujian and all those on Taiwan with the exception of population GS have the lowest nucleotide and haplotype diversity (Table 1).

Pairwise F_{ST} and Nm values (Table 3) indicate high genetic differentiation between the Taiwan and mainland Asia (China and Vietnam) populations. Significant F_{ST} values are found between the Taiwan and China populations ($F_{ST} = 0.97953$), as well as the Taiwan and Vietnam populations ($F_{ST} = 0.95346$). The two Nm values between the Taiwan and mainland Asia (China and Vietnam) populations (0.01045 and 0.02441, respectively) were found to be much lower than the Nm for the China and in Vietnam populations (33.99915). The AMOVA results indicate that 94.51% of the genetic variation in *Taiwania* lies within its geographical distribution (Table 4). When the China and Vietnam populations are considered as one geographical region (mainland Asia), the percentage of variation between mainland Asia and Taiwan was 95.99%, which is slightly higher than the percentage obtained when China and Vietnam are treated as distinct geographical regions.

Phylogeographical relationships among *Taiwania* populations

The statistical parsimony network indicates that the Taiwanese haplotype A and haplotype B differed by eight mutations

Table 4 Hierarchical analysis of molecular variance (AMOVA) based on the cpDNA sequences of *Taiwania*.

Comparison setting	Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation
Among populations in different geographical regions (Taiwan, China and Vietnam)	Among populations in Taiwan, China and Vietnam	2	141.457	1.42860***	94.51
	Within populations in Taiwan, China and Vietnam	150	12.437	0.08291	5.49
	Total	152	153.894	1.51152	
Between the populations in Taiwan and mainland Asia (China and Vietnam)	Between populations in Taiwan and mainland Asia	1	141.260	2.00371***	95.99
	Within populations in Taiwan and mainland Asia	151	12.634	0.08367	4.01
	Total	152	153.894	2.08738	
Between populations in China and Vietnam	Between populations in China and Vietnam	1	0.197	0.00175	1.45
	Within populations in China and Vietnam	96	11.446	0.11923	98.55
	Total	97	11.644	0.12099	

Levels of significance are based on 1000 random permutations with $P < 0.001$ marked by ***.

Table 5 Summary of nested clade phylogeographical analyses (NCPA) for clades of *Taiwania* showing statistically significant geographical association of haplotype distribution.

Clade	Clade being nested	Dc	Dn	Inference chain	Outcome
Total cladogram ($P < 0.0001$)	Clade 3-1 (haplotype A)	47.8089^L	573.8992^L	$1 \rightarrow 19$ NO	Allopatric fragmentation
	Clade 3-2 (haplotype B-H)	619.6679^L	979.2603^S		

See Fig. 1b for clade positions. Superscript S and L indicate that distance measures are significantly smaller and larger, respectively, than expected under random distribution of haplotypes. Dc indicates clade distance, Dn indicates nested clade distance.

(Fig. 1b). These included a single 8-bp deletion gap at 309–316 bp of *pet*G–*trn*P in haplotypes A and I (Table 2). This genetic distance was greater than the differences between each of the haplotypes from mainland Asia (Fig. 1b). On the network, haplotype B was located closer to the outgroup than haplotype A (Fig. 1b). The NCPA results detected only significant geographical association ($P < 0.0001$) for geographical haplotype distribution between populations in Taiwan (clade 3-1) and mainland Asia (clade 3-2) (Table 5). According to the updated NCPA inference key (Templeton, 2004), this result indicates allopatric separation following fragmentation of the ancestral population (Table 5).

Estimate of divergence times

All the results derived from the three statistical methods indicate a mean divergence time between the Taiwan and mainland Asia populations towards the end of the Pliocene (3.23–3.41 Ma) (Fig. 2). MCMCcoal generated the earliest mean divergence time, at 3.41 Ma with a 95% confidence interval (CI) (2.70, 4.19). In contrast, the divergence time of all *Taiwania* populations generated by paml/multidivtime produced the most recent mean divergence time at 3.23 Ma with a 95% CI (2.55, 4.00).

Results also indicated secondary divergences within the mainland Asia haplotypes and within the Taiwanese haplotypes during the early Pleistocene (Fig. 2). For the haplotypes in mainland Asia, the paml/multidivtime generated a mean divergence time of *c.* 1.00 Ma with a 95% CI (0.21, 2.04; Fig. 2), while the TMRCA generated by beast suggests an earlier mean divergence time of 1.39 Ma with a 95% HPD (0.87, 2.01; Fig. 2) after the log combination by Tracer. For the haplotypes in Taiwan, the paml/multidivtime generated a mean divergence time of *c.* 0.80 Ma with a 95% CI (0.09,
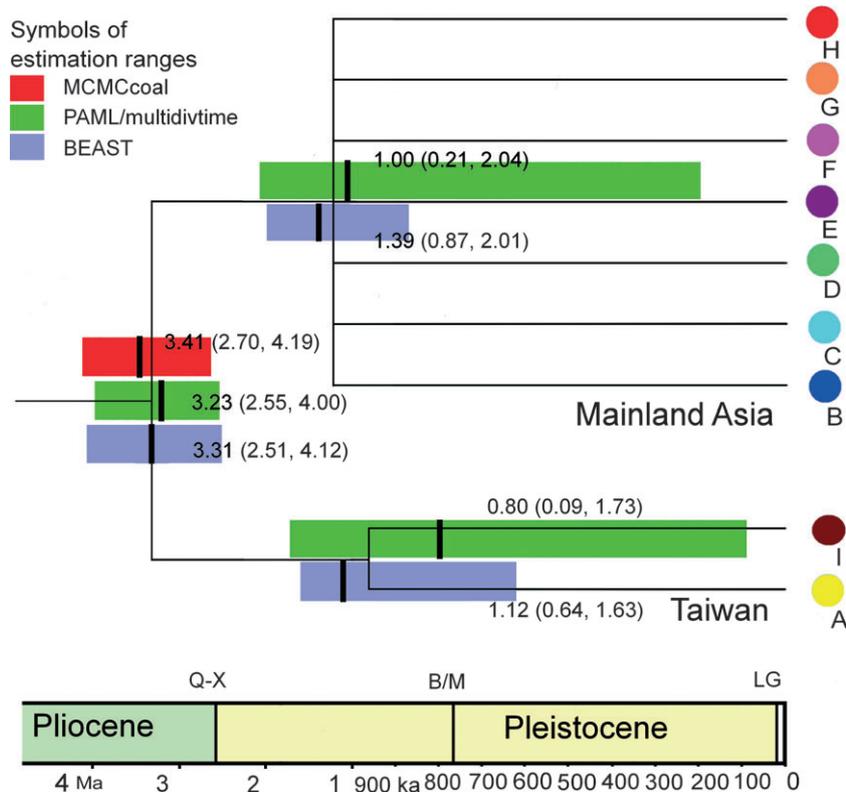
Figure 2 Estimates of divergence time of *Taiwania* populations with fossil calibration. Time ranges of MCMCcoal, paml/multidivtime or beast estimates are indicated by red, green and blue bars, respectively, with black lines marking the means. The upper and lower limits of 95% confidence interval (or highest posterior density) are provided in parentheses. Q-X, onset of the Quaternary; B/M, Brunhes–Matsuyama boundary; LG, Late Glacial Period.

1.73; Fig. 2), while the TMRCA generated by BEAST suggests an earlier mean divergence time of 1.12 Ma with a 95% HPD (0.64, 1.63; Fig. 2) after the log combination by TRACER. The posterior shape of the TMRCAs generated by BEAST had a normal distribution with a slightly positive skew.

DISCUSSION

Low cpDNA genetic diversity of *Taiwania* populations

Only nine cpDNA haplotypes were identified in the *Taiwania* populations analysed. This value is much lower than in other studies of East Asian conifers that have used cpDNA markers: 28 in *C. konishii* (*trn*V intron, *pet*G–*trn*P, *trn*D–*trn*T and *trn*L–*trn*F; Hwang *et al.*, 2003); 19 in *Taxus wallichiana* (*trn*L–*trn*F; Gao *et al.*, 2007); and 19 in *Ginkgo biloba* (*trn*K; Gong *et al.*, 2008).

Nine of the 12 *Taiwania* populations analysed contained a single haplotype ($\pi = 0.00000$, $h = 0.000$, Table 1; Fig. 1a). Of the remaining three populations, the Yunnan–Myanmar population (YC) and the Vietnam population (LV) contained four ($\pi = 0.00015$, $h = 0.298$) and five haplotypes ($\pi = 0.00017$, $h = 0.275$), respectively, while the Guanshan population (GS, Taiwan) contained only two haplotypes ($\pi = 0.00006$, $h = 0.167$). The high frequency of populations containing a single haplotype (nine of 12 populations) is unusual compared with *C. konishii* (10 of 31 populations, Hwang *et al.*, 2003) and the *Taxus wallichiana* complex (16 of 50 populations, Gao *et al.*, 2007). Our results indicate that the extant *Taiwania* populations preserve a low level of cpDNA genetic diversity compared with other East Asian gymnosperms.

Strong genetic differentiation between mainland and island populations

Because conifers are wind-pollinated and their cpDNA is usually paternally inherited, low genetic differentiation between populations is expected (Wagner, 1992; Nybom, 2004; Petit *et al.*, 2005). The absence of shared haplotypes between the mainland Asia and Taiwan populations emphasizes the lack of gene flow between them (Tables 1, 3 & 4; Fig. 1a). This is in contrast to the results of studies on *C. konishii* (Hwang *et al.*, 2003) and the *Pinus luchuensis* complex (Chiang *et al.*, 2006), where the Taiwan and mainland Asia populations, which are separated by similar or greater distances and topographic barriers, share at least one ancestral cpDNA haplotype. Furthermore, compared with the *Taxus wallichiana* complex, in which only a single hypothetical haplotype separated the populations in Taiwan from those in adjacent areas of mainland China (Gao *et al.*, 2007), in *Taiwania* seven hypothetical haplotypes separated the mainland Asia and Taiwan populations (Fig. 1b). This may reflect longer temporal separation of the *Taiwania* populations compared with the *Taxus* populations.

Multiple *Taiwania* refugia along the Yunnan–Myanmar border, Vietnam and Taiwan

Sites of potential refugia are generally predicted to have a widespread ancestral haplotype as well as other mutational derived and unique haplotypes (Crandall & Templeton, 1993). An ancestral haplotype is also expected to occupy a central position within a haplotype network and consequently have more mutational connections to other haplotypes (Crandall & Templeton, 1993). On mainland Asia, haplotype B is predominant, geographically widespread and located in the centre of the haplotype network. As such it is likely to be an ancestral haplotype (Table 2 & Fig. 1b). Two populations contain additional derived and unique haplotypes. The Yunnan–Myanmar population (YC) contains haplotypes C, D and E, while the Vietnam population (LV) contains haplotypes C, F, G and H. On this basis, these populations may represent potential refugia.

The identification of the Vietnam population is interesting, as Vietnam and adjoining provinces of Lao PDR also contain isolated populations of conifers such as *Cunninghamia* and *Glyptostrobus* that have similar palaeohistories to *Taiwania*. However, recent phylogeographical and genetic studies on these taxa did not include samples from Vietnam or Lao PDR in their analyses (Hwang *et al.*, 2003; Li & Xia, 2005) and may have overlooked potential refugia. Further research is needed to test this hypothesis.

Of the remaining *Taiwania* populations, all insular populations with the exception of population GS contain only haplotype A. The mutational difference between A and B indicates a long history of divergence. Additionally, haplotypes A and B are situated at the opposite ends of two independent lineages from the outgroup in the haplotype network (Fig. 2). This implies that haplotype A is also an ancestral haplotype, and on that basis the Taiwanese populations may also represent a potential refugium. The almost total absence of additional haplotypes in the Taiwanese populations could be the result of strong gene flow between populations that are geographically close to each other and where topographic and ecological barriers are minimal. Previous studies using random amplified polymorphic DNA (RAPD) and allozyme markers have also indicated that there has been widespread gene flow and little population differentiation between *Taiwania* populations in Taiwan (Lin *et al.*, 1993; Ju *et al.*, 2006). The presence of an additional haplotype in population GS in the southernmost part of *Taiwania*'s range on Taiwan could indicate the presence of a smaller refugium, although further research is needed to test this hypothesis.

The populations in Fujian, Guizhou and Hubei are fixed for a single, ancestral haplotype and therefore could be considered as potential refugial populations. The presence of a single haplotype could be the result of gene flow, as has been suggested for the populations in Taiwan. However, the large geographical distances and significant topographical barriers between them make this explanation unlikely (Fig. 1). Alternatively, they could be the result of fragmentation of a wider

population and have become fixed at a single haplotype through random genetic drift. This may have been possible if haplotype B was also the most common haplotype in the past. A third alternative is that they are the result of recolonization or human-mediated introduction from putative refugial populations in Yunnan and Vietnam, although in that case they would not be considered refugial. Their status remains unclear.

Divergence times and migration routes of *Taiwania* populations

Our molecular clock estimates indicate that the divergence of the Asian *Taiwania* population into two major lineages, represented by the ancestral haplotypes A and B, occurred during the late Pliocene (3.23–3.41 Ma). This is almost concurrent with *Taiwania*'s disappearance from the fossil record in Japan (Momohara, 1992, 1994, 1999; Momohara & Mizuno, 1999; LePage, 2009) and restriction in the range of numerous East Asian endemic taxa, such as *Amentotaxus*, *Cephalotaxus*, *Cunninghamia*, *Cryptomeria*, *Glyptostrobus*, *Ginkgo*, *Keteleeria*, *Metasequoia* and *Taiwania*, into refugial areas (LePage *et al.*, 2005; LePage, 2009; Manchester *et al.*, 2009). It is also concurrent with increased seasonality and aridity across Southeast Asia as well as global cooling due to accelerated uplift of the Qinghai-Xizang Plateau and Himalayas during the late Pliocene (*c.* 3.4 to 2.6 Ma) (An *et al.*, 1999, 2001; An, 2000); such climate change may have contributed to the fragmentation of endemic populations and their ultimate isolation.

Our estimates also indicate secondary divergence events within the mainland Asia lineage represented by the ancestral haplotype B, and within the Taiwanese lineage between haplotypes A and I, during the early to mid-Pleistocene (Figs 1b & 2). The more recent divergence among the mainland Asia populations is supported by the lower genetic differentiation between the Vietnam and China populations ($F_{ST} = 0.01449$; Table 3). This secondary divergence is almost concurrent with an intensification of the glacial cycles in the middle Pleistocene (Ehlers & Gibbard, 2007).

Our results indicate that, while one lineage migrated to Taiwan during or soon after the late Pliocene, a second spread across mainland China and was eventually restricted to northern Vietnam and the Myanmar–China area during the early Pleistocene. The migration route to Taiwan could have been via a land bridge across the narrow Taiwan Strait that separates Taiwan from mainland China. This strait is only 50–100 m below the current sea level and would have been exposed during the Plio-Pleistocene glacial cycles, when sea levels were lower, allowing for the migration of plants and animals in either direction (Shen, 1994).

An alternative hypothesis for *Taiwania*'s presence in Taiwan is a southward migration via a land bridge along the Japanese Ryukyu Islands. This may have been possible during the Pleistocene via temporary land bridges or island hopping during periods when the climate was cooler. Palynological data indicate that both montane and lowland conifers such as *Abies*, *Cryptomeria*, *Pinus* and *Tsuga* were present in Okinawa until *c.* 1.78 Ma, after which they became extinct (Fujiki & Ozawa, 2008), probably in response to climatic and environmental changes associated with increased global cooling and aridification. However, in the absence of macrofossils on mainland China or the Ryukyu Islands, both hypotheses remain untested.

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