

Phylogeny of *Calocedrus* (Cupressaceae), an eastern Asian and western North American disjunct gymnosperm genus, inferred from nuclear ribosomal nrITS sequences

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ABSTRACT. To resolve relationships between Taiwanese and other Asian species of *Calocedrus* and to infer the causes of the contemporary distribution of the genus, we analyzed 21 individuals sampled from Vietnam, Yunnan (China), Taiwan, and California (USA). Phylogenetic and phylogeographic analyses based on the nrITS sequence data suggested a deep split between Asian and American *Calocedrus*. In addition, our estimated dates of species divergence are consistent with fossil records and geohistorical evidence. Because, in addition to the distinctive morphology between *C. formosana* and *C. macrolepis*, the sequence divergence between them exceeds the interspecific level of divergence between species of *Taxus*, *C. formosana* could be regarded as a newly emerged distinct species. Speciation of the three species of *Calocedrus* studied was evidently shaped by geohistorical vicariant events, mainly allopatric fragmentations.

Keywords: *Calocedrus*; Disjunct distribution; Fossil records; Geohistorical events; nrITS; Phylogeography.

INTRODUCTION

Species of *Calocedrus* Kurz. (Cupressaceae), also known as incense cedars, are characterized by their flat branchlets with strongly decussate and dimorphic leaves, which are composed of two pairs, a small and median facial pair and a larger, narrowly triangular lateral one (Figure 1). The pollen bearing cones of incense cedars are solitary; the ovule bearing ones are solitary (or sometimes in a pair in *C. formosana*) ovoid-oblong with three pairs of scales, of which the upper pair is united in a flat plate between the outer two pairs. The lowermost (or outermost) pair is short, reduced and reflexed, and only the middle pair is fertile. The seeds have two distinctly unequal wings (Krüssmann, 1985; Page, 1990). Four species have been recognized in *Calocedrus*. They have a typical eastern Asia-western North American disjunct pattern of distribution (Guo, 1999; Xiang et al., 1998; Xiang et al., 2001) (Figure 2). The three eastern Asian taxa are *C. macrolepis* Kurz., *C. formosana* (Florin) Florin, and *C. rupestris* Aver., H.T. Nguyen & L.K. Phan. *Calocedrus*

macrolepis is indigenous to southwestern China (including Hainan Island), northern Vietnam, and Myanmar; *C. formosana* is endemic to Taiwan (Krüssmann, 1985; Page, 1990; Li and Keng, 1994); and *C. rupestris* is endemic to northern Vietnam (Averyanov et al., 2008). The fourth taxon, *C. decurrens* (Torr.) Florin, is native to the Cascade Mountains of Oregon and the Sierra Nevada of California and extends into Baja California (Krüssmann, 1985; Page, 1990).

Fossil records indicate that in the Tertiary *Calocedrus* not only occurred in eastern and southeastern Europe, but also in more northern regions than it does today. For example: Kvaček and Hably (1998) and Kvaček (1999) recognized a fossil collection of *C. suleticensis* from the early Oligocene of the Czech Republic and Hungary and the early Miocene of Greece and another fossil species, *C. pliocenica*, from the Pliocene (5.3-1.8 million years ago [MYA]) of Poland. Wolfe (1972) described a leafy branch of the genus from the Oligocene-Miocene boundary (23.8 MYA) of Alaska. Kvaček (1999) also found *C. schornii* from the Oligocene of Oregon and *C. masonii* from the Miocene of Idaho. Additionally, Liu and Zheng (1995), Kvaček and Hably (1998), and Kvaček (1999) reported *C. lantenoisii* from the Miocene of Yunnan

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Province (China) and *C. notoensis* from Japan. No fossils of the genus have been recorded from western Europe or eastern North America.

Of the four incense cedars, the taxonomic status of *C. formosana* has been exceptionally controversial. It was first attributed to *Libocedrus macrolepis* when it was reported from Taiwan by Hayata (1908). Because of its smaller and obtuse lateral leaves (see Figure 1 in this study), 3-anthered stamens, and larger cones (pollen bearing cones 4-6 mm long and ovule bearing cones 12-15 mm long) and seeds (5 mm long), *C. formosana* was considered different from plants on the mainland by Florin (1930), thus it was recognized as *L. formosana*. Kudo (1931), however, claimed that the characters used by Florin were variable and not sufficiently significant to recognize the Taiwanese plants as a distinct species. He therefore treated it as *L. macrolepis* var. *formosana* (Florin) Kudo. At that time *Calocedrus* was included in *Libocedrus*, which is now limited to species native to New Zealand and New Caledonia. After the recognition of *Calocedrus* by Li (1953), Florin (1956)

made the combination, *C. formosana* (Florin) Florin, for the Taiwanese incense cedar. Interestingly, western taxonomists preferred to treat *C. formosana* as a distinct species (e.g. Florin, 1956; Krüssmann, 1985; Kvaček, 1999) while eastern taxonomists (e.g. Li and Keng, 1994) followed Kudo. Hence, a new approach to resolving these conflicting views is needed.

Sequence data from the internal transcribed spacers of nuclear ribosomal DNA (nrITS) have been utilized successfully to elucidate phylogenetic relationships at the generic and specific levels of both gymnosperms (e.g. Liston et al., 1996; Cheng et al., 2000) and angiosperms (e.g. Shi et al., 1998; Wen and Shi, 1999). Here we present our comparative analyses of nrITS from 21 individuals of three taxa of *Calocedrus*, *C. decurrens*, *C. formosana*, and *C. macrolepis*. Relationships within the genus were inferred and discussed based on the sequence variation and reconstructed phylogenetic trees. Furthermore, phylogeographic inferences and fossil records were used to determine the historical scenarios that might account for the present disjunct distribution of *Calocedrus*.

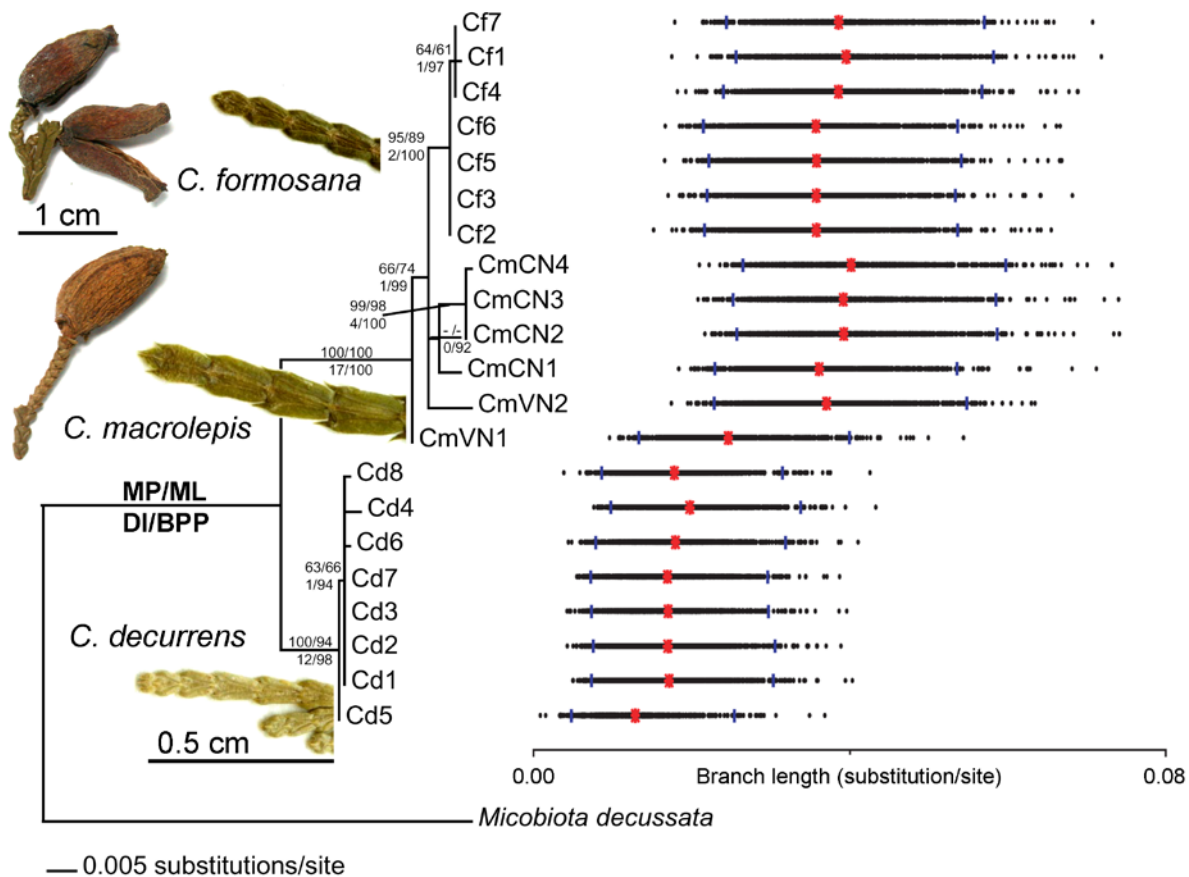


Figure 1. ML phylogeny for *Calocedrus* (-lnL = 2379.00157; model: TrN + Γ). Branch support was calculated using MP bootstrapping (above branch before slash), ML bootstrapping (above branch after slash), Decay index (below branch before slash), and BPP (below branch after slash). Bayesian relative rate (BRR) test is shown next to individual codes; orange points indicate MLEs; and blue lines at both edges illustrate 95% BPPs. Estimated values overlap 95% BPPs, indicating a constant evolutionary rate. Left figures illustrate leaf branches for three species of *Calocedrus* (scale bar = 0.5 cm) and ovulate cones for *C. formosana* and *C. macrolepis* (scale bar = 1 cm); *C. formosana* and *C. macrolepis* (HAST accessions 65530 and 43827, respectively).

MATERIALS AND METHODS

Sampling

Nr ITS sequences from a total of 21 individuals of three species of *Calocedrus* were obtained (Table 1). All vouchers were deposited in the herbarium of Taiwan Endemic Species Research Institute (TAIE) except for the Chinese samples, which were gathered from cultivated individuals in the Atlanta Botanical Garden (The original collecting information for the Chinese species of *Calocedrus* is given in Table 1). GenBank nrITS sequences from *Microbiota decussata*, *C. macrolepis* and *C. formosana* were downloaded and included in our analysis. Based on the chloroplast *rbcL* gene (Brunsfeld et al., 1994) and combined molecular and morphological data (Gadek et al., 2000), *Microbiota decussata* was selected as the outgroup because it was shown to be one of the closest sisters of *Calocedrus* and has a published nrITS sequence. Herbarium specimens examined are HAST accessions 24005, 35634, 59941, 62772, 65530, 67179, 94644, 101265, 101266 (*C. formosana*, all from Taiwan),

and 43827 (*C. macrolepis*, from Yunnan, China).

DNA extraction and sequencing

Genomic DNA was extracted from fresh leaves or leaves dried in silica gel following the method of Shure et al. (1983). We designed the PCR primers based on conserved regions of two published GenBank accessions, U77962 and U77954 (both *Cupressus arizonica*). The forward primer CUP1 (5' GGTATTCACGCCTGACTTGG3') is located at the 3' end of *18S rRNA* gene, and the reverse one CUP2 (5' ATAGGTGAACCTGCGGTAGG3') is at the beginning sequence of the *26S rRNA* gene. The PCR products were purified using GeneClean II (Bio 101, CA) and subcloned into a pGEM T-Easy vector (Promega, WI). Plasmid DNA was purified using a Qiaprep Spin Miniprep Kit (Qiagen, Hilden, Germany). Sequencing was performed using an ABI377 automated sequencer with Bigdye™ Terminator Cycle Sequencing Ready Reaction Kit (PE Biosystems, CA). For each individual, at least two independent PCR clones were sequenced.

Table 1. Analyzed individuals and their corresponding accession numbers.

Species	Codes ^a	Vouchers	Accessions	Sampling Localities
<i>Microbiota decussata</i>			AY380874	Little et al., 2004
<i>C. decurrens</i>	Cd1	Chen 3227	AY150679	Lloyd Meadows Basin, Tulare County, California, USA
	Cd2	Chen 3228	AY150680	Lloyd Meadows Basin, Tulare County, California, USA
	Cd3	Chen 3229	AY150681	Lloyd Meadows Basin, Tulare County, California, USA
	Cd4	Chen 3231	AY150682	Lloyd Meadows Basin, Tulare County, California, USA
	Cd5	Chen 3232	AY150683	Lloyd Meadows Basin, Tulare County, California, USA
	Cd6	Chen 3233	AY150684	Lloyd Meadows Basin, Tulare County, California, USA
	Cd7	Chen 3234	AY150685	Lloyd Meadows Basin, Tulare County, California, USA
	Cd8		AY380854	Little et al., 2004
<i>C. macrolepis</i>	CmVN ^b 1	Chen 4161	AY150686	Dalat, Vietnam
	CmVN ^b 2	Chen 4162	AY150687	Dalat, Vietnam
	CmCN ^b 1	ABG 98-0764	AF287249	Mekong Salween Divide, Yunnan, China; Cultivated in Atlanta Botanical Garden
	CmCN ^b 2	ABG 97-1416	AY150688	Kunming Inst. of Botany, Yunnan, China; Cultivated in Atlanta Botanical Garden
	CmCN ^b 3	ABG 97-1422	AY150689	An Fen Ying, Yunnan, China; Cultivated in Atlanta Botanical Garden
	CmCN ^b 4	ABG 97-1421	AY150690	An Fen Ying, Yunnan, China; Cultivated in Atlanta Botanical Garden
<i>C. formosana</i>	Cf1	Chen 3539	AY150691	Ching-Shui Village, Chung-Liao, Nantou, Taiwan
	Cf2	Chen 4163	AY150692	Chi-Chi Township, Nantou, Taiwan
	Cf3	Chen 4164	AY150693	Chi-Chi Township, Nantou, Taiwan
	Cf4	Chen 4159	AY150694	Fusing village, Taoyuan, Taiwan
	Cf5	Chen 4160	AY150695	Fusing village, Taoyuan, Taiwan
	Cf6	Chen 3540	AF287248	Ching-Shui Village, Chung-Liao, Nantou, Taiwan
	Cf7		AY380855	Little et al., 2004

^aCodes were abbreviations of the scientific names and numbers of individuals.

^bVN and CN represent individuals of *C. macrolepis* from Vietnam and China, respectively.

Sequence alignment and phylogenetic analyses

Sequences were aligned using the Clustal W implemented in the MegAlign program (DNASTAR, Inc.) with manual inspection. The best fit sequence evolution model was selected using both the Akaike Information Criterion (AIC) and Bayesian Information Criterion (BIC) by MODELTEST (version 3.7, Posada and Crandall, 1998). Phylogeny was reconstructed based on the selected molecular evolution model using the maximum likelihood (ML) method implemented in PAUP* (version 4.0b10, Swofford, 2001). An ML heuristic search was performed using a neighbor joining (NJ) starting tree and tree bisection and reconnection (TBR) swapping strategy.

Maximum parsimony (MP) branch support was done using a non-parametric bootstrap (Felsenstein, 1985) with ten random sequence additions, TBR branch swapping, and 1000 replicates using PAUP*. ML branch support was carried out using 1000 replicates with the first iteration using an NJ starting tree (TBR branch swapping). The decay index (DI, Bremer, 1988) was first calculated by TreeRot (version 3, Sorenson and Franzosa, 2007) using the obtained MP topologies to generate a PAUP* command file and then implemented in PAUP*. The Bayesian Posterior Probability (BPP) for branches was calculated using MrBayes (version 3.1.2, Huelsenbeck and Ronquist, 2001). The sequences obtained were partitioned into three regions, *ITS1*, *5.8S*, and *ITS2*, using “charset,” and each region was given the most complicated model (nst=6, rate=invgamma) using “applyto.” Two independent runs were performed simultaneously, and each run contained four Markov chains. The Markov Chain Monte Carlo (MCMC) searches were run for 1×10^6 generations, with chains being sampled every 100 generations. We calculated the Bayes factor between the two runs to investigate whether these two runs converged at the end of the analysis. The initial 1,000 trees were discarded as burn-in. The post-burn-in trees were imported into PAUP* to compute the 50% majority rule trees.

Rate constancy and estimation of divergence time

A Bayesian relative rate (BRR) test was conducted using Cadence (version 1.08beta, Wilcox et al., 2004). *Microbiota decussata* was selected as the outgroup for calculating the branch lengths from all ingroup individuals to the most recent common ancestor (MRCA). Significantly different evolutionary rates were identified if the 95% posterior distributions of calculated branch lengths did not overlap with each other.

Estimation of divergence times of nodes using the Bayesian network was carried out using PAML (version 4, Yang, 2007) and MULTIDIVTIME (version 9/25/03, Thorne and Kishino, 2002) with the F84 + Γ model (kept 1×10^5 samples, samples taken every 100 cycles, and the first 1×10^6 cycles discarded as burnin). The root of *Calocedrus* was constrained between 33.7 and 23.8 MYA because fossil records of the genus first emerged

and became abundant during the Oligocene. One ML ultrametric tree which assumed a molecular clock was obtained by “multidivtime” and visualized using MEGA (version 4, Tamura et al., 2007).

Phylogeographic analysis

Nested clade analysis (NCA, Templeton et al., 1995; Templeton, 2008) was carried out first using TCS (version 1.21, Clement et al., 2000) to construct the most parsimonious network for those obtained sequences with a 95% confidence interval. This network was then nested using the rules of Templeton et al. (1992). GeoDis (version 2.5, Posada et al., 2000) was used to calculate the clade distance (D_c) and nested clade distance (D_n) for those designed nested clades. Once the D_c and/or D_n was/were detected as significant, the inference key (Templeton, 2004) was applied to discriminate effects of different types of historical events—restricted gene flows, past fragmentation, and range expansion—that may have resulted in correlation of sequence divergence and spatial and/or temporal factors.

RESULTS

Sequence variation

The amplified nrITS sequences comprised the *ITS1*, *5.8S rRNA*, and *ITS2* regions. Thirteen unique haplotypes were found within the 21 individuals of *Calocedrus*. The 19 newly-determined *Calocedrus* sequences were deposited in GenBank (Table 1). The lengths of the nrITS sequences were 1,067 bp in *C. decurrens*, 1,088 bp in the Asian mainland *C. macrolepis*, and 1,087 bp in the Taiwanese *C. formosana* before alignment. Gene boundaries were identified by comparison with other GenBank available sequences, e.g. *Taiwania* and *Taxus* (Cheng et al., 2000). The *ITS1* regions have the highest length variations, which are due to a 34 bp deletion in the accessions from western North America, an 11 bp deletion in those from Asia, and one further deletion in those of *C. formosana*. The *5.8S* regions are 145 bp long. The *ITS2* regions are 219 bp long in *C. decurrens* and 218 bp in both *C. macrolepis* and *C. formosana*.

Phylogenetic analyses

TrN + Γ (-lnL = 2381.512) was selected as the best fit evolutionary model for the nrITS regions using both AIC (4775.0239) and BIC (4805.1665). The ML phylogeny (Figure 1) indicated that Asian and American *Calocedrus* were each monophyletic and that the sampled *C. macrolepis* was paraphyletic if we treated the *C. formosana* as a distinct species. The tree topologies also strongly suggest that the Taiwanese accessions comprise a monophyletic lineage with robust support (95/89/2/100) and is sister to the accessions of the Chinese *C. macrolepis*. Individuals of *C. macrolepis* from Vietnam occupied the basal position on the Asian *Calocedrus* lineage. Twice the deviation in the harmonic means was 1.8 between the

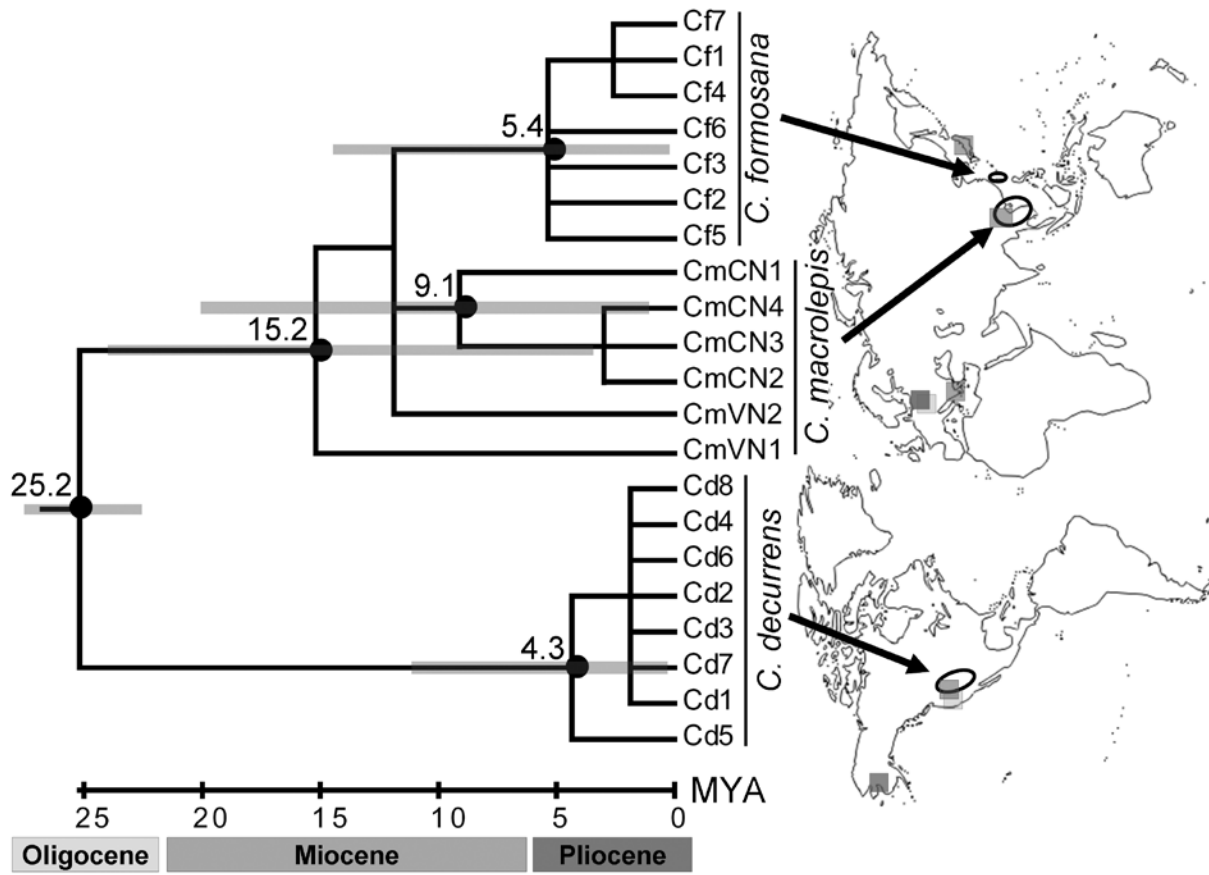


Figure 2. Bayesian estimations of divergence times for three species of *Calocedrus* using known fossil records for calibration. Numbers before nodes denote estimated divergence times; gray bars represent 95% CIs. Diagram below time scale denotes corresponding geological period; shaded boxes and scaled horizontal lines represent locations and geological time for fossils of *Calocedrus*. Circles show current distribution of *Calocedrus*.

two Bayesian runs, indicating that the difference between two runs is not statistically significant (Kass and Raftery, 1995).

Rate constancy and the estimation of divergence time

A BRR test (Figure 1) showed an overlapping 95% BPP for the analyzed data, which implies constant evolutionary rates within the nrITS regions of the samples of *Calocedrus* that were examined. Our estimated time of divergence between Asian and American *Calocedrus* is 25.2 (28.9-23.8) MYA. The divergence time is 4.3 (11.8-0.6) MYA for the American individuals, 15.2 (24.4-4.6) MYA for Asian *Calocedrus*, 9.1 (19.4-1.7) MYA for the Chinese *C. macrolepis*, and 5.4 (14.6-0.5) MYA for *C. formosana* (Figure 2).

Phylogeographic analysis

A most parsimonious network was generated from the analyzed data (Figure 3). The Asian species of *Calocedrus* are connected to one another using 95% CI, as are the American haplotypes. Nevertheless, the connection

between Asian and American lineages should be extended to 81 steps. D_c and/or D_n were detected to be statistically significant within most of the clade and nested clade levels. The geohistorical events inferred for the Asian *Calocedrus* clade and the total cladogram were allopatric fragmentations (Figure 3).

DISCUSSION

Two specific deletions in the aligned nrITS sequences of the *Calocedrus* accessions we examined could easily distinguish the Asian and North American taxa and also the two Asian ones. The estimated nrITS pairwise sequence divergence using Kimura's 2-parameter (K2P) distance (Kimura, 1980) among the species of *Taxus* was 0.18% to 1.87% (Li et al., 2001); the pairwise K2P distance between *C. macrolepis* and *C. formosana*, however, was 0.67-1.31%. Considering the similar evolutionary rates of the nrITS region between *Taxus* [ca. 5×10^{-10} base/site/year in *Taxus* (Li et al., 2001)] and *Calocedrus* (4.5×10^{-10} - 5.1×10^{-10}), the sequence divergence between *C. macrolepis* and *C. formosana* exceeds the interspecific level of the species of *Taxus*. Hence, the recognition of

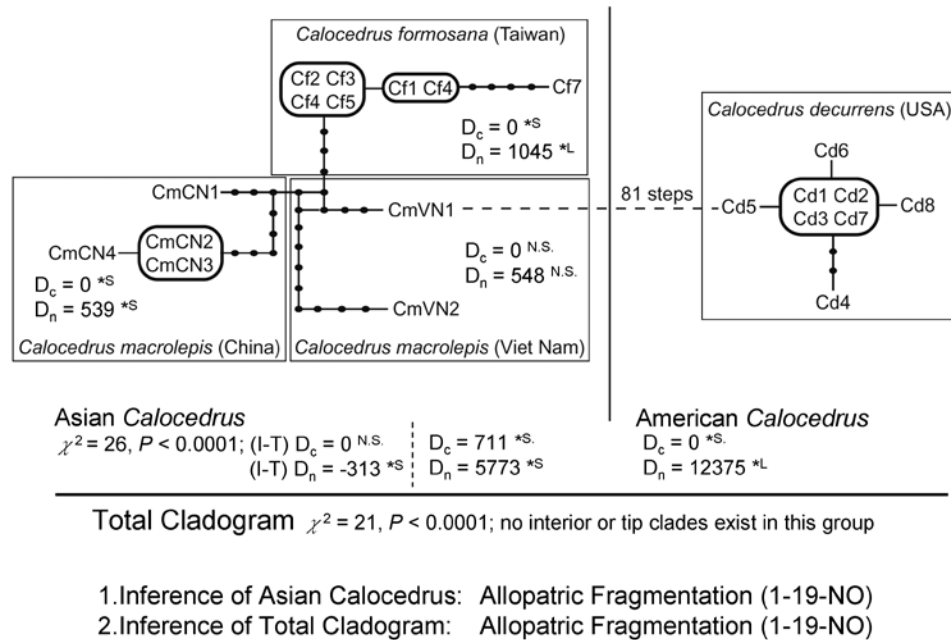


Figure 3. The most parsimonious network of 13 haplotypes (21 individuals) estimated using TCS and the nested clade design. Individuals with same haplotype are combined in an oval box. Geographic coordinates obtained using GoogleEarth are shown next to clades. D_c and D_n and statistical significance are shown within clades (*L: significantly large; *S: significantly small). Allopatric fragmentations are inferred to be major geohistorical events that shaped current distribution of the *Calocedrus*.

C. formosana at specific rank, as proposed by Florin, based on differences in leaf size and shape and cone size appears justified. It is worthy to note that *C. formosana* is also easily distinguished from *C. macrolepis* by its thicker leaves (see also Krüssmann, 1985) and cones with apparently shorter pedicels (Figure 1).

The comparatively long branches revealed by ML phylogeny support the monophylies and distinctiveness of the American and Asian clades (Figure 1), which clearly indicate that the Asian and American lineages have fully diverged. Some plant species are thought to be undifferentiated across continents during an isolation time of about 8 MY (Mayr, 2001). The phylogeographic study of *Tsuga* (Havill et al., 2008) and our present study, however, suggest that the two continental clades have genetically as well as morphologically diverged after a long period of isolation. Since *C. formosana* was resolved to be monophyletic with strong support, it should be treated as distinct from *C. macrolepis* of the Asian mainland (Figure 1).

Despite the findings in a number of molecular studies that some disjunct congeneric species of seed plants between Taiwan, China, and the Ryukyu Islands are paraphyletic (Huang et al., 2001; Lu et al., 2001; Chiang and Schaal, 2006; Chiang et al., 2006), our nrITS data found *Calocedrus* on Taiwan to be monophyletic. The divergence pattern of in *Calocedrus* is similar to that in *Amentotaxus argotaenia*, as revealed by ISSR (Ge et al., 2005). Multiple introduction to Taiwan have been hypothesized for many plant species (Huang et al., 2001;

Lu et al., 2001; Chung et al., 2004; Chiang et al., 2006), and a sorting event could have resulted in discordant phylogenies if organelle and nuclear markers were used (Chiang et al., 2004). Given the relatively short geological history of Taiwan (Liu et al., 2000; Sibuet and Hsu, 2004; Huang et al., 2006), a nuclear marker other than nrITS used here, might better reflect the speciation processes of plants in Taiwan.

The time of divergence of the Asian and the Chinese species of *Calocedrus* is estimated to be during the Miocene, the period from which fossils are known in Yunnan (Figure 2). Previously, no fossils of *Calocedrus* have been reported in Taiwan, but the divergence time of the *C. formosana* is estimated to be around 5 MYA (Figure 2), approximately just after the island of Taiwan emerged above sea level (Sibuet and Hsu, 2004). Fossils provide direct evidence of the existence of species at a given place in the past (Norell and Novacek, 1992; Ren, 1999; Wellman et al., 2003; Xiao et al., 2005), and geohistorical events are limited to species that have existed for a certain period of time in a particular place (Schindewolf et al., 1993; Mueller-Dombois and Fosberg, 1997). A reasonable estimate of divergence time reinforces the robustness of our *Calocedrus* phylogeny.

The contemporary genetic structure of *Calocedrus* was obviously shaped by allopatric fragmentation (Figure 3). Recent studies have demonstrated the importance of selection over pure isolation on speciation (Wu, 2001; Osada and Wu, 2005; Städler et al., 2008). Nevertheless, species of *Calocedrus* are forest trees with life spans

in excess of 20 years. Their highly diverged sequence structure, distinctive morphological characteristics, and discontinuous and localized distributions provide support for allopatric speciation (Hoskin et al., 2005; Nakazato et al., 2007). A more or less contiguous forest distribution throughout the northern Hemisphere was proposed during the Tertiary period (65–2 MYA) via the Bering land bridge (Tiffney, 1985a; 1985b). With the gradual fragmentation of habitats as the earth's climate fluctuated, plants and animals became isolated and diverged into distinct species (Xiang et al., 1998; Xiang and Soltis, 2001; Von Dohlen et al., 2002; Havill et al., 2008; Sanmartín et al., 2008). Speciation within formerly connected and widespread species of *Calocedrus* through fragmentations of the range of the genus could have been the result of global climate changes during that period.

CONCLUSION

The present study, with support from fossil and geohistorical evidence, clearly demonstrates the genetic uniqueness of the three species of *Calocedrus*. The North American and the Asian species of *Calocedrus* are each monophyletic. Geohistorical events have obviously impacted the diversification of these species. *Calocedrus formosana* was derived from an Asian ancestor and appears to be newly emerged.

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根據核糖體基因間片段重建分佈於亞洲東部及北美西部肖楠屬的親緣關係

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我們分析 21 個來自越南、雲南、台灣及加州的肖楠個體之核糖體基因間片段，探討分布在台灣及亞洲大陸個體之間的關係，並推論導致本屬植物目前分佈型式的原因。我們的分析結果都顯示產於亞洲與美洲的肖楠彼此之間分化相當明顯。除此之外估算出來的物種分化時間皆與化石出現時間或地理事件發生時間一致。台灣肖楠與亞洲大陸產之翠柏之間除了外觀上明顯的形態差異之外，兩者間分子層面的差異亦超出另一個屬（紅豆杉屬）所估算出的物種間差異，故台灣肖楠應被視為一個新形成的獨立物種。而歷史上的不連續分佈的地理事件為造成本研究所選取的三個物種異域種化的機制。

關鍵詞：肖楠；不連續分佈；化石紀錄；nrITS；親緣地理；地理事件。