



Molecular characterization of *Calocedrus rupestris* Aver., H.T. Nguyen & L.K. Phan, 2008 (Cupressaceae) based on ITS1 partial sequence

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ABSTRACT. *Calocedrus rupestris* Aver., H.T. Nguyen & L.K. Phan was described in 2008 based on some morphological characters that were not sufficiently significant to discriminate it as a species distinct from *C. macrolepis* Kurz. We applied a new approach to resolve these conflicting views by using sequence data from DNA (ITS) to elucidate phylogenetic relationships between the two species. Analyses of a partial ITS1 sequence in 5 individuals of 2 subpopulations of *C. macrolepis* and 18 individuals of 8 subpopulations of *C. rupestris* collected in Vietnam were done. Molecular characterization of the two species showed its low divergence with the lack of autapomorphic characters. In addition, the ITS1 partial sequences of some *C. rupestris* individuals were identical with *C. macrolepis*. Due to the less distinctive morphology between *C. rupestris* and *C. macrolepis*, the divergence between them does not exceed the interspecific levels, and therefore, *C. rupestris* could not be regarded as an independent species in relation to *C. macrolepis* but only

as one of its varieties, *C. macrolepis* var. *rupestris* (Aver., H.T. Nguyen & L.K. Phan) L.K. Phan, Long K. Phan & Aver.

Key words: *Calocedrus*; *C. macrolepis*; *C. rupestris*; ITS1; *C. macrolepis* var. *rupestris*

INTRODUCTION

In Vietnam, the genus *Calocedrus* Kurz, comprises 2 recognized species, namely *Calocedrus macrolepis* Kurz and *C. rupestris* Aver., H.T. Nguyen & L.K. Phan. *Calocedrus rupestris* differs from *C. macrolepis* only by the stalk of seed cones being much shorter with less imbricate scales than in *C. macrolepis* (0.5-1-(1.5) mm vs 3-(5) mm length, 6-8-(12) vs 10-20 scales). Other characteristics to separate this species from *C. macrolepis* were variable and overlapped (such as the white abaxial stomatal bands, the number of scales in seed cones, and the umbo on fertile seed scales) (Averyanov et al., 2008), which were not sufficiently significant to recognize it as a distinct species. Hence, a new approach to resolving these conflicting views is needed, and the relationship between these species needs to be clarified.

Recently, sequence data from the internal transcribed spacers of nuclear ribosomal DNA (ITS) have been successfully used to elucidate phylogenetic relationships at the specific as well as interspecific levels of *Calocedrus* (Chen et al., 2009), in which ITS1 is highly variable (Liston et al., 1996). In this paper, we present our analyses of a partial ITS1 sequence in 5 individuals of 2 subpopulations of *C. macrolepis* and 18 individuals of 8 subpopulations of *C. rupestris* collected in Vietnam.

MATERIAL AND METHODS

Sampling

A total of 43 leaf samples of *C. rupestris* and *C. macrolepis* were collected in Vietnam, in which *C. rupestris* samples were collected from 13 sites in 4 provinces and *C. macrolepis* were collected from 3 sites in 1 province (Table 1). Each sample was kept in a Falcon tube with silica gel and stored at -70°C. In this study, we chose 18 samples of *C. rupestris* and 5 samples of *C. macrolepis*, according to the sampling sites, for sequencing. The remaining samples were deposited at the Vietnam National Museum for Nature and The New York Botanical Garden.

DNA extraction and sequencing

Total DNA was extracted from dried leaves using the DNeasy Plant Minikit (Qiagen, Singapore). The forward primer CITSF (3'CTT AAC CGT GTA GGG GAT GGA G5') and the reverse primer CITSR (3'ACT TGA TGA TTC ACG GGA TTC TGC5') were designed based on the ITS sequence of the genus *Calocedrus* obtained from GenBank and used in the PCR reaction for amplification of the partial ITS1 and partial 5.8S sequences. After purification, DNA fragments were sequenced with a BigDye Terminator Cycle Sequencing Ready Reaction kit (PE Applied Biosystems, Foster City, CA, USA) and run on an ABI PRISM 3100 Genetic Analyzer (PE Applied Biosystems).

Table 1. *Calocedrus* spp samples in Vietnam.

No.	Vouchers	Species	Locality	Sequence type*
1	P 10893	<i>C. rupestris</i>	Ha Giang: Quan Ba, Can Ty, 23°05'06" N, 105°01'56" E, ca. 1300 m	1
2	P 10895	<i>C. rupestris</i>	Ha Giang: Quan Ba, Thai An, around point 23°05' N, 105°03' E, 1300 m	2
3	P 10909	<i>C. rupestris</i>	Ha Giang: Meo Vac, Pai Lung, 23°15'14" N, 105°22'35" E, ca. 1300 m	2
4	P 10910	<i>C. rupestris</i>	Ha Giang: Meo Vac, Pai Lung, 23°15'14" N, 105°22'35" E, ca. 1300 m	2
5	P 10919	<i>C. rupestris</i>	Son La: Yen Chau, Muong Lum, 21°01'18" N, 104°29'29" E, ca. 1000 m	2
6	P 10921	<i>C. rupestris</i>	Son La: Yen Chau, Muong Lum, 21°01'18" N, 104°29'29" E, ca. 1000 m	3
7	P 10926	<i>C. rupestris</i>	Son La: Yen Chau, Muong Lum, Ban On Oc, 21°01'18" N, 104°29'29" E, ca. 1000 m	3
8	P 10928	<i>C. rupestris</i>	Son La: Yen Chau, Muong Lum, Ban Lum, 21°01'18" N, 104°29'29" E, ca. 1000 m	2
9	P 10930	<i>C. rupestris</i>	Son La: Yen Chau, Muong Lum, Ban Lum, 21°01'18" N, 104°29'29" E, ca. 1000 m	1
10	PVT 542	<i>C. rupestris</i>	Bac Kan: Na Ri, Liem Thuy, Na Po, 21°56'44" N, 106°05'09" E, 700 m	1
11	PVT 544	<i>C. rupestris</i>	Bac Kan: Na Ri, Liem Thuy, Na Po, 21°56'44" N, 106°05'09" E, 700 m	2
12	P 10938	<i>C. rupestris</i>	Quang Binh: Bo Trach, A Rem, Frontier Military Station, 17°23'32" N, 106°12'46" E, ca. 700 m	4
13	P 10941	<i>C. rupestris</i>	Quang Binh: Bo Trach, A Rem, Frontier Military Station, 17°23'32" N, 106°12'46" E, ca. 700 m	4
14	P 10943	<i>C. rupestris</i>	Quang Binh: Bo Trach, A Rem, between Frontier Military Station and HQ of A Rem Comm., 17°23'32" N, 106°12'46" E, 700 m	4
15	P 10945	<i>C. rupestris</i>	Quang Binh: Bo Trach, A Rem, between Frontier Military Station and HQ of A Rem Comm., 17°23'32" N, 106°12'46" E, 700 m	2
16	P 10948	<i>C. rupestris</i>	Quang Binh: Bo Trach, A Rem, HQ of A Rem Comm. 17°23'32" N, 106°12'46" E, 600 m	2
17	P 10949	<i>C. rupestris</i>	Quang Binh: Bo Trach, A Rem, HQ of A Rem Comm. 17°23'32" N, 106°12'46" E, 600 m	4
18	P 10953	<i>C. rupestris</i>	Quang Binh: Bo Trach, A Rem, km 37, 17°23'32" N, 106°12'46" E, 600 m	2
19	P 10958	<i>C. macrolepis</i>	Lam Dong: Da Lat city, Hang Cop watershed, 11°56'49" N, 108°33'08" E, ca. 1400 m	2
20	P 10961	<i>C. macrolepis</i>	Lam Dong: Da Lat city, Hang Cop watershed, 11°56'49" N, 108°33'08" E, ca. 1400 m	5
21	P 10965	<i>C. macrolepis</i>	Lam Dong: Da Lat city, S of Dan Ta La watershed, 11°53'17" N, 108°27'42" E, ca. 1300 m	1
22	P 10966	<i>C. macrolepis</i>	Lam Dong: Da Lat city, S of Dan Ta La watershed, 11°53'17" N, 108°27'42" E, ca. 1300 m	6
23	P 10970	<i>C. macrolepis</i>	Lam Dong: Da Lat city, Dan Ta La watershed, 11°54'04" N, 108°26'56" E, ca. 1300 m	6

*Sequences with same number are identical.

Data analysis

Sequences of *Calocedrus* spp from Vietnam were aligned using Clustal X 1.64 (Thomson et al., 1997) with sequences of *Calocedrus* spp, according to Chen et al. (2009). Equally weighted maximum parsimony (MP) and maximum likelihood (ML) analyses were performed using PAUP* (4.0 beta version) (Swofford, 1998). A heuristic search procedure was used with the following settings: ten replicates of random taxon addition, tree-bisection reconnection branch swapping, multiple trees retained, no steepest descent, and accelerated transformation. Gaps were treated as missing data. Bootstrap analysis was carried out with 100 replicates. For ML analysis, the appropriate substitution model of DNA evolution that best fitted the data set was determined by the Akaike Information Criterion with MODEL Test 3.7 (Posada and Crandall, 1998). Bootstrap analysis with 100 replicates was conducted to assess the degree of support for ML tree clades.

RESULTS

Relationship with other species

The ITS1 partial sequences of the ITS region were obtained from 23 individuals of *Calocedrus* from Vietnam; however, some sequences were identical (Table 1). Therefore we selected 6 sequences representing 6 sequence types for further analysis.

The length of the *Calocedrus* spp ITS1 partial sequence for alignment with an out-group taxon was 715 bp. MP analysis of this alignment indicated that among 715 characters, 42 were parsimony informative.

The phylogenetic trees obtained from MP (tree length 145) and ML (DNA model = TrN+G, -ln likelihood = 1680.4764) had similar topology (Figure 1).

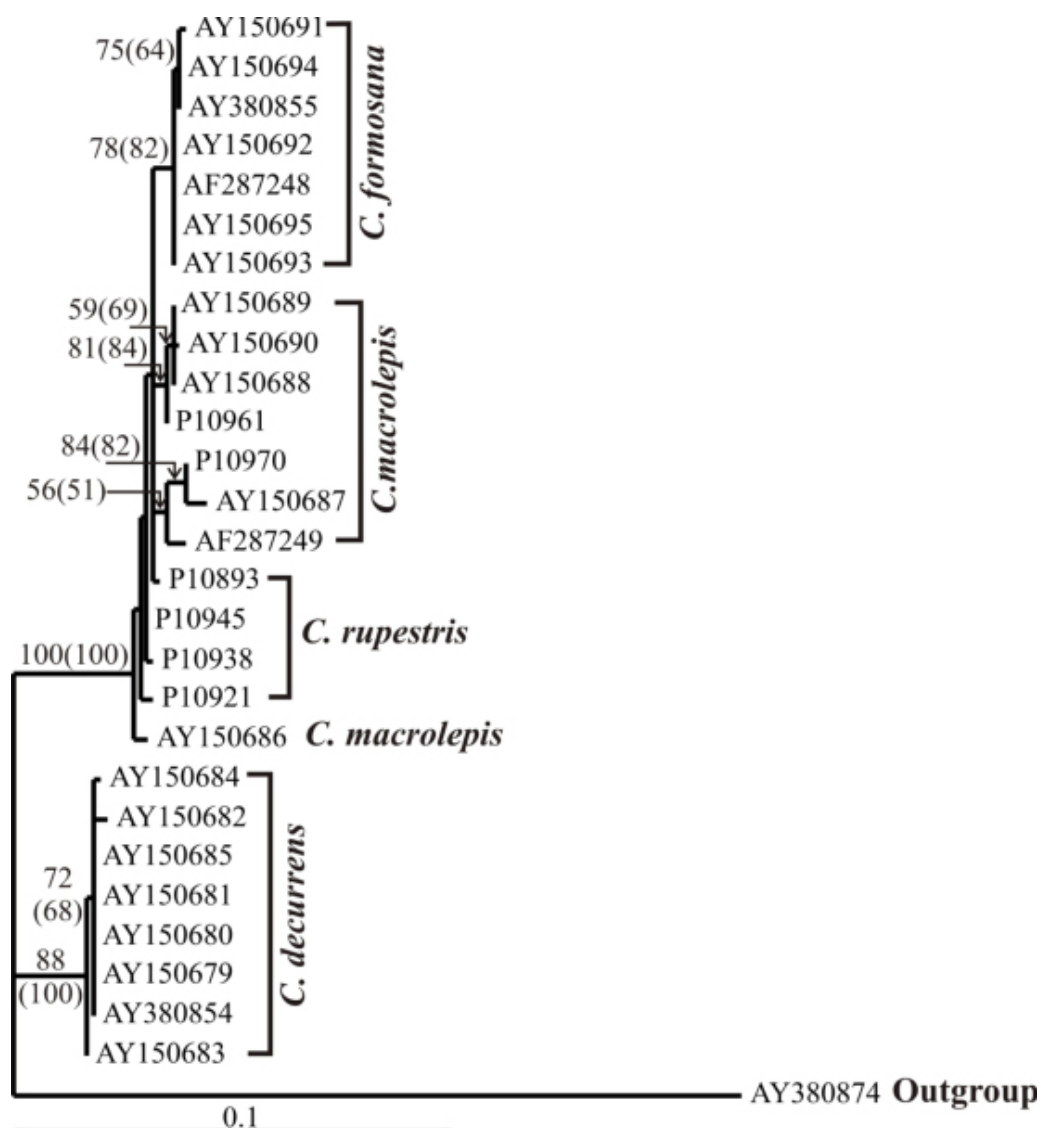


Figure 1. A single maximum likelihood tree (Ln likelihood = -1680.4764, TrN+G model of DNA evolution) obtained from analysis of the alignment of six Vietnamese *Calocedrus* spp with other sequences of *Calocedrus* spp (Chen et al., 2009). Bootstrap values are given in appropriate clades. Bootstraps for MP are in brackets. Scales indicate the number of nucleotide changes.

Our results based on ITS1 partial sequences support the same evolutionary lines previously found by Chen et al., (2009) on the basis of all ITS sequences. Two clades of Asian and American *Calocedrus* as well as the position of *C. macrolepis* (AY150686) from Dalat, Vietnam located at the basal position of the Asian *Calocedrus* lineage were observed in both analyses. However, some of our results differed from the previous study. *Calocedrus macrolepis* (AY15087), which was located at the basal position of the Asian *Calocedrus* lineage (Chen et al., 2009), clustered with *C. macrolepis* (P10970), which was also collected from Dalat, Vietnam with moderate bootstrap support (ML = 84% and MP = 82%) and had a sister relationship with *C. macrolepis* (AF287249) (bootstrap ML = 56% and MP = 51%), and this clade had a sister relationship with the clade of *C. macrolepis* (P10961) and *C. macrolepis* (AY150688, AY150689, AY150690). The clade of *C. formosana* (bootstrap support ML = 78% and MP = 82%) had a sister relationship with the clades of *C. macrolepis*. All *C. rupestris* individuals were clustered in the Asian clade with strong bootstrap support (100%) and appeared as a sister taxon of *C. macrolepis* and *C. formosana*.

Sequence variation

From the phylogenetic tree, we selected *C. macrolepis* from China (AY150689, AF287249) and Vietnam (AY150686, AY150687), *C. formosana* (AY150691, AY150692), *C. decurrens* (AY150683, AY150684), which represented each clade in the variable sequence comparison. The ITS1 partial sequence of Vietnamese *Calocedrus* was 681 bp, the same as for *C. macrolepis* from China (AY150689, AF287249) and Vietnam (AY150686, AY150687) and longer than that of *C. formosana* (AY150691, AY150692) and *C. decurrens* (AY150683, AY150684) (Figure 2). The ITS1 partial sequences of *C. rupestris* differed from each other, from 1 (*C. rupestris* P10938 vs *C. rupestris* P10945) to 5 nucleotides (*C. rupestris* P10893 vs *C. rupestris* P10921) and from that of other species from 3 (*C. rupestris* P10893 vs *C. macrolepis* P10961) to 32 nucleotides (*C. rupestris* P10893 vs *C. decurrens* AY150684). The pairwise divergence between taxa ranged from 0.1 to 5.8 (Table 2). *Calocedrus decurrens* was clearly distinguished from other species by 74 autapomorphic characters.

DISCUSSION

Currently, the genus *Calocedrus* comprises 3 species, namely *C. macrolepis*, *C. formosana* and *C. decurrens* (Farjon, 2005). This is a genus with a disjunct area of distribution: western North American with only *C. decurrens* and the remaining species in Eastern Asia. In 2008, with the newly described *C. rupestris*, the total number of species of this genus increased to 4 species, 3 of which are distributed in Asia (Averyanov et al., 2008). Some authors such as Cheng and Fu (1978) followed the concept of Kudo and reduced *C. formosana* (Florin) Florin to a variety of *C. macrolepis*, but Chen et al. (2009) newly retained it as distinct species. Currently, the genus *Calocedrus* consists of 4 species, including *C. macrolepis*, *C. formosana* and *C. rupestris*. *Calocedrus macrolepis* is distributed in southern China including Taiwan, India, Myanmar, Thailand, and Vietnam, where it is found on the silicate mountains in Dak Lak, Khanh Hoa, Lam Dong and probably Ninh Thuan (in the south), with very few available records in Bavi Mountain, Hanoi City (in the north). *Calocedrus rupestris* is only recorded in Vietnam and is considered an endemic species. This species is distributed in limestone mountains in the northern part of Vietnam, and it may also be found along the Chinese and Laotian borders (Averyanov et al., 2008).

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      *      20      *      40      *
C. formo. AY150691 : CCGCATGCCGAGCTCGTCTCTT-CCCGGCCCGGAGATCTCAGCGACGTGGGAC : 54
C. formo. AY150692 : .....-..... : 54
C. macro. AY150689 : .....C..... : 54
C. macro. P10961 : .....-..... : 54
C. macro. P10970 : .....-..... : 54
C. macro. AY150687 : .....-..... : 54
C. macro. AF287249 : .....-..... : 54
C. macro. AY150686 : .....-..... : 54
C. rupestris. P10893 : .....-..... : 54
C. rupestris. P10945 : .....-..... : 54
C. rupestris. P10938 : .....-..... : 54
C. rupestris. P10921 : .....-..... : 54
C. decurrens. AY150684 : .....C...T..... : 48
C. decurrens. AY150683 : .....C...T..... : 48

      60      *      80      *      100      *
C. formo. AY150691 : GCCTGGCCCTCGCAGTCCCGTGTGGCGGCAAGGCCTCAAGCGCACGGCGITGG : 109
C. formo. AY150692 : ..... : 109
C. macro. AY150689 : .....C..... : 109
C. macro. P10961 : .....C..... : 109
C. macro. P10970 : .....T..... : 109
C. macro. AY150687 : .....T..... : 109
C. macro. AF287249 : .....T.....C..... : 109
C. macro. AY150686 : .....T..... : 109
C. rupestris. P10893 : .....C..... : 109
C. rupestris. P10945 : .....C..... : 109
C. rupestris. P10938 : .....C..... : 109
C. rupestris. P10921 : .....C..... : 109
C. decurrens. AY150684 : .....A...A..... : 76
C. decurrens. AY150683 : .....A...A..... : 76

      120      *      140      *      160
C. formo. AY150691 : GGTCCAGGCTGATCGAATGCCGCGGTCGAATGTGTTCCTTCCCGCCG-GGGGAGG : 163
C. formo. AY150692 : .....-..... : 163
C. macro. AY150689 : .....G..... : 164
C. macro. P10961 : .....G..... : 164
C. macro. P10970 : .....G..... : 164
C. macro. AY150687 : .....A.....G..... : 164
C. macro. AF287249 : .....G..... : 164
C. macro. AY150686 : .....G..... : 164
C. rupestris. P10893 : .....G..... : 164
C. rupestris. P10945 : .....G..... : 164
C. rupestris. P10938 : .....G..... : 164
C. rupestris. P10921 : .....G..... : 164
C. decurrens. AY150684 : .....A.....A.....G.....A : 131
C. decurrens. AY150683 : .....A.....G.....A : 131

      *      180      *      200      *      220
C. formo. AY150691 : A-CCCGGATCGAATGCCGATGCAATCGGTGCCTGCCACGACGTTTACGTGGG : 217
C. formo. AY150692 : .....-..... : 217
C. macro. AY150689 : .....-..... : 218
C. macro. P10961 : .....-..... : 218
C. macro. P10970 : .....-..... : 218
C. macro. AY150687 : .....-..... : 218
C. macro. AF287249 : .....-..... : 218
C. macro. AY150686 : .....-.....T..... : 218
C. rupestris. P10893 : .....-..... : 218
C. rupestris. P10945 : .....-..... : 218
C. rupestris. P10938 : .....-..... : 218
C. rupestris. P10921 : .....-T.....T..... : 218
C. decurrens. AY150684 : GA.....A...T..... : 186
C. decurrens. AY150683 : GA.....A...T..... : 186

      *      240      *      260      *
C. formo. AY150691 : AGCCAGTGGTCTGCTCGGTTGCGAGGAGAGATCCCGGCCCTGCCCGGCTC : 272
C. formo. AY150692 : ..... : 272
C. macro. AY150689 : ..... : 273
C. macro. P10961 : ..... : 273
C. macro. P10970 : ..... : 273
C. macro. AY150687 : ..... : 273
C. macro. AF287249 : .....A..... : 273

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Figure 2. Multiple sequence alignment of the partial ITS1 sequence of selected *Calocedrus* spp: *C. formo* = *C. formosana*; *C. macro* = *C. macrolepis*; *C. rupestris* = *C. rupestris*; *C. decur* = *C. decurrens*.

Continued on next page

Figure 2. Continued.

C. macro. AY150686	:	:	273
C. rupea. P10893	:	:	273
C. rupea. P10945	:	:	273
C. rupea. P10938	:T.....	:	273
C. rupea. P10921	:	:	273
C. decur. AY150684	:	...T.....T	:	241
C. decur. AY150683	:	...T.....T	:	241
		200 * 300 * 320 *		
C. formo. AY150691	:	GAGGCCGCACGCATCGCGATCGCGTGCAGCGCGCTGTGCCAGGGATCCGCGT	:	327
C. formo. AY150692	:	:	327
C. macro. AY150689	:	:	328
C. macro. P10961	:	:	328
C. macro. P10970	:	:	328
C. macro. AY150687	:T.....	:	328
C. macro. AF287249	:	:	328
C. macro. AY150686	:	:	328
C. rupea. P10893	:	:	328
C. rupea. P10945	:	:	328
C. rupea. P10938	:	:	328
C. rupea. P10921	:	:	328
C. decur. AY150684	:CT.....	:	296
C. decur. AY150683	:CT.....	:	296
		340 * 360 * 380		
C. formo. AY150691	:	CGATGGCGCAAGTCGGGATCGCCCAACCCCGCTCGTGTGGATCGTGGGTAA	:	382
C. formo. AY150692	:	...C.....	:	382
C. macro. AY150689	:	...C.....	:	383
C. macro. P10961	:	...C.....	:	383
C. macro. P10970	:	...C.....	:	383
C. macro. AY150687	:	...C.....	:	383
C. macro. AF287249	:	...C.....C.....	:	383
C. macro. AY150686	:	...C.....	:	383
C. rupea. P10893	:	...C.....	:	383
C. rupea. P10945	:	...C.....	:	383
C. rupea. P10938	:	...C.....	:	383
C. rupea. P10921	:	...C.....	:	383
C. decur. AY150684	:	...C.....T.....G.....G.....C.....	:	351
C. decur. AY150683	:	...C.....T.....G.....G.....C.....	:	351
		* 400 * 420 * 440		
C. formo. AY150691	:	CTCGTGGCNCGGAGGTTCCGTGTTCTGGATGGGAGCACGCGCATGACTGCGCGT	:	437
C. formo. AY150692	:	:	437
C. macro. AY150689	:	:	438
C. macro. P10961	:	:	438
C. macro. P10970	:	:	438
C. macro. AY150687	:	:	438
C. macro. AF287249	:	:	438
C. macro. AY150686	:	:	438
C. rupea. P10893	:	:	438
C. rupea. P10945	:	:	438
C. rupea. P10938	:	:	438
C. rupea. P10921	:	:	438
C. decur. AY150684	:T.....A.....T.....	:	406
C. decur. AY150683	:T.....A.....T.....	:	406
		* 460 * 480 *		
C. formo. AY150691	:	GGTAGGACCCCGATCCGGTGGCAGGCTCTCCGCGCGGCGAGTCCCACTCAG	:	492
C. formo. AY150692	:G.....	:	492
C. macro. AY150689	:A.....G.....G.....	:	493
C. macro. P10961	:A.....G.....G.....	:	493
C. macro. P10970	:T.....G.....G.....	:	493
C. macro. AY150687	:T.....G.....G.....	:	493
C. macro. AF287249	:G.....G.....	:	493
C. macro. AY150686	:G.....G.....	:	493
C. rupea. P10893	:G.....G.....	:	493
C. rupea. P10945	:G.....G.....	:	493
C. rupea. P10938	:G.....G.....	:	493
C. rupea. P10921	:G.....G.....	:	493
C. decur. AY150684	:C.....G.....T.....	:	461
C. decur. AY150683	:C.....G.....T.....	:	461

Continued on next page

Figure 2. Continued.

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      500      *      520      *      540      *
C. formo. AY150691 : OGACGGGACGGGGCGGGGACACACCGGAGCGTTCCTTCGGTGCCACCCG--GAGCGC : 545
C. formo. AY150692 : ..... : 545
C. macro. AY150689 : ...G..... : 546
C. macro. P10961 : ...G..... : 546
C. macro. P10970 : ...GC..... : 546
C. macro. AY150687 : ...GC..... : 546
C. macro. AP287249 : ...G..... : 546
C. macro. AY150686 : ...G..... : 546
C. rupes. P10893 : ...G..... : 546
C. rupes. P10945 : ...G..... : 546
C. rupes. P10938 : ...G..... : 546
C. rupes. P10921 : ...G..... : 546
C. decur. AY150684 : ...G.....C.....A...TC.G.T.. : 516
C. decur. AY150683 : ...G.....C.....TC.G.T.. : 516

      560      *      580      *      600
C. formo. AY150691 : -----GTGCGGGACGTGTCAACACCCCAACACACGGGTGCATCAGCACCTC : 591
C. formo. AY150692 : ----- : 591
C. macro. AY150689 : ----- : 592
C. macro. P10961 : ----- : 592
C. macro. P10970 : ----- : 592
C. macro. AY150687 : ----- : 592
C. macro. AP287249 : ----- : 592
C. macro. AY150686 : -----T----- : 592
C. rupes. P10893 : ----- : 592
C. rupes. P10945 : ----- : 592
C. rupes. P10938 : ----- : 592
C. rupes. P10921 : ----- : 592
C. decur. AY150684 : CCGGAGCGC.....T.....C. : 571
C. decur. AY150683 : CCGGAGCGC.....T.....C. : 571

      *      620      *      640      *      660
C. formo. AY150691 : AGAAATCCAAACATTGAAAAGTGCAGATCGATCGCCCTTGCAGGGTCCGGTCCGACG : 646
C. formo. AY150692 : ..... : 646
C. macro. AY150689 : .....C..... : 647
C. macro. P10961 : .....C..... : 647
C. macro. P10970 : ..... : 647
C. macro. AY150687 : .....G..... : 647
C. macro. AP287249 : ..... : 647
C. macro. AY150686 : .....T..... : 647
C. rupes. P10893 : .....A..... : 647
C. rupes. P10945 : .....T..... : 647
C. rupes. P10938 : .....T..... : 647
C. rupes. P10921 : .....T..... : 647
C. decur. AY150684 : G.....T..A..... : 625
C. decur. AY150683 : G.....T..A..... : 625

      *      680      *
C. formo. AY150691 : AAAGAGAGAGACATMCGAAATCAGACTCTCGG : 680
C. formo. AY150692 : ..... : 680
C. macro. AY150689 : ..... : 681
C. macro. P10961 : ..... : 681
C. macro. P10970 : ..... : 681
C. macro. AY150687 : .....C..... : 681
C. macro. AP287249 : .....C..... : 681
C. macro. AY150686 : ..... : 681
C. rupes. P10893 : ..... : 681
C. rupes. P10945 : ..... : 681
C. rupes. P10938 : ..... : 681
C. rupes. P10921 : ..... : 681
C. decur. AY150684 : .....A..... : 659
C. decur. AY150683 : .....A..... : 659

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Table 2. Pairwise distance between taxa in *Calocedrus* (below diagonal: total character differences, above diagonal: mean character differences adjusted for missing data).

No.	Species	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1	<i>C. formosana</i> AY150691	-	0.00294	0.01176	0.01029	0.01618	0.01176	0.01176	0.01176	0.00882	0.00882	0.01029	0.01324	0.05426	0.05116
2	<i>C. formosana</i> AY150692		-	0.00882	0.00735	0.01324	0.00882	0.00882	0.00882	0.00588	0.00588	0.00735	0.01029	0.05116	0.04806
3	<i>C. macrolepis</i> AY150689			-	0.00147	0.01615	0.01175	0.01175	0.01175	0.00587	0.00587	0.00734	0.01028	0.05263	0.04954
4	<i>C. macrolepis</i> PI0961				-	0.01468	0.01028	0.01028	0.01028	0.00441	0.00441	0.00587	0.00881	0.05108	0.04799
5	<i>C. macrolepis</i> PI0970					-	0.00587	0.01028	0.00734	0.00734	0.00734	0.00881	0.01175	0.05263	0.04954
6	<i>C. macrolepis</i> AY150687						-	0.01322	0.01322	0.01322	0.01322	0.01468	0.01762	0.05882	0.05573
7	<i>C. macrolepis</i> AF287249							-	0.01175	0.00881	0.00881	0.01028	0.01322	0.05263	0.04954
8	<i>C. macrolepis</i> AY150686								-	0.00881	0.00587	0.00734	0.00734	0.04489	0.04180
9	<i>C. rupestris</i> PI0893									-	0.00294	0.00441	0.00734	0.04954	0.04644
10	<i>C. rupestris</i> PI0945										-	0.00147	0.00441	0.04644	0.04334
11	<i>C. rupestris</i> PI0938											-	0.00587	0.04799	0.04489
12	<i>C. rupestris</i> PI0921												-	0.04489	0.04180
13	<i>C. decurrens</i> AY150684													-	0.00303
14	<i>C. decurrens</i> AY150683														-

Morphologically, *C. rupestris* differs from *C. macrolepis* in some minor characters (Averyanov et al., 2008). Our analysis of ITS1 partial sequences showed that the sequence divergence within *C. rupestris* ranged from 0.0 to 0.7% and overlapped with that of *C. macrolepis* (0.0 to 1.76%). The divergence between the two species was low (from 0.0 to 1.76%), with the lack of autapomorphic characters. In addition, our sequencing results showed that the partial ITS1 sequence of some *C. rupestris* individuals was identical to that of *C. macrolepis* individuals (i.e., *C. rupestris* P10893, 10930, PVT542 were identical to *C. macrolepis* P10965 and *C. rupestris* P10895, P10909, P10910, P10919, P10928, PVT544 were identical to *C. macrolepis* P10958) (see Table 1). In addition to the less distinctive morphology between *C. rupestris* and *C. macrolepis*, the divergence between them does not exceed the interspecific level, and therefore, *C. rupestris* could not be regarded as an independent species in relation to *C. macrolepis* but only as one of its varieties, *C. macrolepis* var. *rupestris* (Aver., H.T. Nguyen & L.K. Phan) L.K. Phan, Long K. Phan & Aver.

Although *C. formosana* can be distinguished from *C. macrolepis* by a few morphological characters, i.e., its thicker leaves and cones with apparently shorter pedicels (Chen et al., 2009), and differs from other species by 2 autapomorphic characters demarcating *Calocedrus* from Taiwan (Figure 2), the divergence within *C. formosana* is low (from 0.0 to 0.29%) in the range of that of *C. macrolepis*. Therefore, *C. formosana* could be considered a variety of *C. macrolepis*.

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