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## A transcriptome-based resolution for a key taxonomic controversy in Cupressaceae

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3 **Title:**

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5 **A transcriptome-based resolution for a key taxonomic controversy in Cupressaceae**

6

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20 **Running title:** A transcriptome-based phylogeny for junipers and cypresses

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## 24 ABSTRACT

25 **Background and Aims** Rapid evolutionary divergence and reticulate evolution may result in  
26 phylogenetic relationships that are difficult to resolve using small nucleotide sequence datasets.  
27 Next-generation sequencing methods can generate larger datasets that are better suited to solving  
28 these puzzles. One major and long-standing controversy in conifers concerns generic  
29 relationships within the subfamily Cupressoideae (105 species, ~1/6 of all conifers) of  
30 Cupressaceae, in particular the relationship between *Juniperus*, *Cupressus* and the  
31 *Hesperocyparis-Callitropsis-Xanthocyparis* (HCX) clade. Here we attempt to resolve this  
32 question using transcriptome-derived data.

33 **Methods** Transcriptome sequences of 20 species from Cupressoideae were collected. Using  
34 MarkerMiner, single copy nuclear (SCN) genes were extracted. These were applied to estimate  
35 phylogenies based on concatenated data, species trees, and a phylogenetic network. We further  
36 examined the effect of alternative backbone topologies on downstream analyses, including  
37 biogeographic inference and dating analysis.

38 **Results** Based on the 73 SCN genes (>200,000 bp total alignment length) we considered, all  
39 tree-building methods lent strong support for the relationship (HCX, (*Juniperus*, *Cupressus*));  
40 however, strongly supported conflicts among individual gene trees was also detected. Molecular  
41 dating suggests that these three lineages shared a most recent common ancestor ~60 Mya, and  
42 that *Juniperus* and *Cupressus* diverged ~56 Mya. Ancestral area reconstructions (AARs) suggest  
43 an Asian origin for the entire clade, with subsequent dispersal to North America, Europe and  
44 Africa.

45 **Conclusions** Our analysis of SCN genes resolves a controversial phylogenetic relationship in the  
46 Cupressoideae, a major clade of conifers, and suggests that rapid evolutionary divergence and  
47 incomplete lineage sorting likely acted together as the source for conflicting phylogenetic  
48 inferences between gene trees and between our robust results and recently published studies.  
49 Our updated backbone topology has not substantially altered molecular dating estimates relative

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50 to previous studies, however application of the latest AAR approaches has yielded a clearer  
51 picture of the biogeographic history of Cupressoideae.

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55 **Key words:** single-copy nuclear genes, transcriptome, Cupressoideae, *Hesperocyparis*,

56 *Cupressus*, *Juniperus*, *Xanthocyparis*, *Callitropsis*

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**57 INTRODUCTION**

58  
59 It can be challenging to accurately reconstruct deep phylogenetic relationships within groups that  
60 experienced rapid evolutionary divergence, incomplete lineage sorting and/or reticulate evolution,  
61 especially with small datasets (Maddison, 1997; Dunn *et al.*, 2008; Jian *et al.*, 2008; Zeng *et al.*,  
62 2014; Ruhsam *et al.*, 2015). Rapid evolutionary divergence may lead to short internodal  
63 distances and soft polytomies (Weisrock *et al.*, 2005; Whitfield & Lockhart, 2007; Jian *et al.*,  
64 2008; Pyron *et al.*, 2014; Leaché *et al.*, 2016). In addition, incomplete lineage sorting, which  
65 involves mis-sorting of ancestral polymorphisms relative to the species tree, or reticulate  
66 evolution, which involves the combination or transmission of genetic material between divergent  
67 evolutionary lineages due to hybridization and introgression, may both cause inaccurate or  
68 conflicting species-tree inference (Beiko *et al.*, 2008; Sun *et al.*, 2015).

69 Next-generation sequencing approaches, which generate large amounts of DNA sequence data  
70 from throughout the genome, are transforming phylogenetic inference (e.g. Dunn *et al.*, 2008;  
71 Lee *et al.*, 2011; Faircloth *et al.*, 2012; Zeng *et al.*, 2014). This is especially true where rapid  
72 evolutionary events resulted in few fixed substitutions between divergent species, yielding gene  
73 trees that are usually unresolved with respect to the true species tree, when only a few loci are  
74 used (Whitfield & Lockhart, 2007). A larger amount of sequence data is likely to capture such  
75 species-specific substitutions, potentially resulting in improved phylogenetic resolution (Jian *et*  
76 *al.*, 2008; Zeng *et al.*, 2014). In the case of incomplete lineage sorting, many independent gene  
77 trees from throughout the genome can be used to estimate a credible species tree by reconciling  
78 genealogical discordance between loci (Edwards, 2009; Lemmon and Lemmon, 2013). Therefore,  
79 phylogenetic estimation of species trees based on genomic datasets might resolve branches that  
80 were poorly supported in smaller datasets (Rokas *et al.*, 2003; Dunn *et al.*, 2008). For example,  
81 phylogenetic analyses using as few as 29 and 59 low-copy nuclear genes have resulted in  
82 well-resolved deep phylogenetic estimates for ferns (Rothfels *et al.*, 2015) and flowering plants  
83 (Zeng *et al.*, 2014), respectively.

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84 Two main methods have recently been proposed to construct species trees from large datasets  
85 (Liu *et al.*, 2009a, 2009b, 2015). One method uses the multiple-species coalescent model as  
86 implemented in the program \*BEAST (Heled and Drummond, 2010), which estimates gene trees  
87 and the species tree at the same time. However, this method is computationally intensive  
88 (Edwards *et al.*, 2007; Pyron *et al.*, 2014), and may result in poor convergence if the dataset is  
89 large (O'Neill *et al.*, 2013). The other method uses a two-step approach when estimating species  
90 trees. In the first step gene trees are generated using software such as RaxML (Stamatakis *et al.*,  
91 2014), and in the second step they are summarized under the coalescent model as implemented in  
92 the software MP-EST (Liu *et al.*, 2010), STAR (Liu *et al.*, 2009a). This method reduces  
93 computation time considerably when compared to analyses based on the multiple species  
94 coalescent model (Liu *et al.*, 2009b). In addition, a recently developed two-step approach,  
95 ASTRAL-II (Mirarab *et al.*, 2015; Mayyari and Mirarab, 2016), has been shown to run much  
96 faster and to be less sensitive than MP-EST to the effects of gene tree errors, when estimating a  
97 species tree based on large dataset (e.g. hundreds of taxa and thousands of genes). The accuracy  
98 of ASTRAL remains high when a small number of genes is adopted and a moderate level of  
99 incomplete lineage sorting is assumed, whereas its local posterior probabilities of quartet  
100 branches are conservative; this leads to very few false positives that have high support, at the  
101 cost of missing some true positives (Mayyari and Mirarab, 2016).

102 Cupressaceae, also known as the cypress family, contains more than 160 species in 32 genera,  
103 of which 17 are monotypic (Farjon, 2005; Mao *et al.*, 2012; Yang *et al.*, 2012; Wang & Ran,  
104 2014; Adams 2014). They occur in many different habitats on all continents except Antarctica  
105 (Farjon, 2005). Cupressoideae, which contains more than 100 species in 13 genera, is the largest  
106 of the seven subfamilies of Cupressaceae (Gadek *et al.*, 2000; Mao *et al.*, 2012; Yang *et al.*,  
107 2012). This subfamily occurs throughout the Northern Hemisphere and contains many  
108 ecologically important and dominant species especially in mountainous and arid or semi-arid  
109 regions (Farjon, 2005; Adams 2014). It contains many economically important timber species  
110 (e.g. *Calocedrus*, *Chamaecyparis*, *Cupressus* and *Thuja*) and ornamental trees (e.g.

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111 *Chamaecyparis*, *Juniperus*, *Platycladus* and *Thuja*) (Farjon, 2005). Phylogenetic analyses  
112 suggest that this subfamily is monophyletic (Gadek *et al.*, 2000; Mao *et al.*, 2012; Yang *et al.*,  
113 2012) and comprises four clades (Gadek *et al.*, 2000; Little 2006; Mao *et al.*, 2012; Yang *et al.*,  
114 2012) which have been treated as separate tribes by some authors (Gadek *et al.*, 2000). However,  
115 taxonomic treatment at the generic level and inter-generic relationships within the subfamily  
116 remains controversial (Little *et al.*, 2004; Little 2006; Mill & Farjon, 2006; Rushforth, 2007;  
117 Adams *et al.*, 2009; Christenhusz *et al.*, 2011; Dörken *et al.*, 2017), especially for *Cupressus*  
118 *sensu lato* (s.l.), which comprises 30 species (Little, 2006; Christenhusz *et al.*, 2011; Dörken *et*  
119 *al.*, 2017). *Cupressus* s.l. may be divided into four genera: *Cupressus sensu stricto* (s.s.) and  
120 *Xanthocyparis* s.s. in the Old World, and *Hesperocyparis* and *Callitropsis* s.s. in the New World  
121 (Adams *et al.*, 2009; Mao *et al.*, 2010; Christenhusz *et al.*, 2011) (see Table 1 for a summary of  
122 taxonomic treatment history). Henceforth, if not stated otherwise, “*Cupressus*”, “*Xanthocyparis*”,  
123 and “*Callitropsis*” refer to *Cupressus* s.s., *Xanthocyparis* s.s. and *Callitropsis* s.s., respectively.  
124 Although the monophyly of *Cupressus* and the *Hesperocyparis-Callitropsis-Xanthocyparis* clade  
125 (the HCX clade; Mao *et al.*, 2010) is well defined (Little *et al.*, 2004; Little 2006; Mao *et al.*,  
126 2010, 2012; Yang *et al.*, 2012), the phylogenetic relationship between *Cupressus*, the HCX clade  
127 and *Juniperus* remains uncertain. All possible phylogenetic topologies among these three clades  
128 have been supported by different studies with different datasets and analyses, as follows:  
129 (*Cupressus*, (*Juniperus*, HCX)) topology was recovered by Xiang & Li (2005), Adams *et al.*  
130 (2009) and Terry & Adams (2015); (*Juniperus*, (*Cupressus*, HCX)) topology by Mao *et al.*  
131 (2010); (HCX, (*Cupressus*, *Juniperus*)) topology by Little (2006) and Yang *et al.* (2012); and a  
132 trichotomy (HCX, *Cupressus*, *Juniperus*) by Mao *et al.* (2012). From here on, these topologies  
133 are referred to as Cu(HCX,Ju), Ju(Cu,HCX), HCX(Cu,Ju) and (HCX,Cu,Ju), respectively, for  
134 simplicity. A recent phylogenomic study based on the whole plastid genomes of 22 species of  
135 Cupressaceae and accounting for long branch attraction (e.g., Felsenstein, 1978; Hendy and  
136 Penny, 1989) supported the Ju(Cu,HCX) topology (Qu *et al.*, 2017). However, all of these  
137 studies either used no more than four bi-parentally inherited nuclear loci (e.g. Little 2006; Adams



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138 *et al.*, 2009) or plastid DNA (ptDNA), the latter of which, despite the use of nine (Mao *et al.*,  
139 2010), 11 ptDNA regions (Terry & Adams, 2015) or even the whole plastid genome (Qu *et al.*,  
140 2017), can be considered to be a single locus due to its lack of recombination.

141 The aim of the current study is to resolve this long-standing controversy and to reconstruct the  
142 phylogenetic relationship between *Cupressus*, *Juniperus* and the HCX clade based on a number  
143 of single or low copy nuclear loci from transcriptome data using 17 species representing major  
144 lineages within these three clades, plus three outgroups. Specifically, we investigate (a) the  
145 evolutionary relationship between the three major lineages using a phylotranscriptomic approach,  
146 (b) compare and explain the discordance and agreement between the current species tree  
147 topology and phylogenetic topologies that were gained in previous studies, and characterize the  
148 impact of different topologies of the three major lineages on (c) molecular dating of this group  
149 and (d) the inference of its biogeographic history.

150

## 151 **MATERIALS AND METHODS**

152

### 153 **Provenance of samples**

154

155 Fresh leaf samples from a total of 18 species (including outgroup species *Microbiota decussata*)  
156 were collected for transcriptome sequencing. Fourteen samples were collected from the living  
157 collection of the Royal Botanic Garden Edinburgh (RBGE), three were collected in the field in  
158 Yunnan, China (*Cupressus duclouxiana*) and Xizang, China (*Cupressus gigantea* and *Juniperus*  
159 *microsperma*), and one (*Cupressus funebris*) was a cultivated individual from the campus of  
160 Sichuan University, Chengdu, China (Table 2). Additionally, we used transcriptome data for  
161 three outgroup species (*Calocedrus decurrens*, *M. decussata*, *Thuja plicata*) from the one  
162 thousand transcriptome project ('1000 plant project,' 1KP) (Table 2). All species were  
163 represented by a single accession apart from *M. decussata* (n=2).

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## 165 **Transcriptome sequencing, assembly, and alignment**

166  
167 Transcriptomes were either generated in Edinburgh/UK (RBGE, Table 2) or Chengdu/China  
168 (SZ, Table 2) apart from three downloaded from the 1KP project  
169 (<http://www.onekp.com/samples/list.php>; labelled as ‘1kp’ in Table 2). RNA was extracted using  
170 the Spectrum Plant Total RNA Kit (Sigma-Aldrich, St. Louis, Missouri, USA) following  
171 protocol A with a few minor modifications (2-3 times the amount of lysis buffer, 750 µl binding  
172 buffer and three final washes). Library preparation and sequencing was outsourced to Edinburgh  
173 Genomics (Edinburgh, UK) and Novogene (Beijing, China) for RBGE and SZ samples,  
174 respectively (Table 2). Transcriptomes were sequenced on Illumina HiSeq platforms generating  
175  $2 \times 100$  bp paired-end reads. Raw reads were prepared for assembly using Trimmomatic (Bolger  
176 *et al.*, 2014) with the parameters ‘LEADING:3 TRAILING:3 SLIDINGWINDOW:4:15  
177 MINLEN:36’ and cutadapt (Martin 2011) to remove adapters and low quality sequences. Reads  
178 for each taxon were then assembled into contigs with SOAPdenovo-trans (Xie *et al.*, 2014)  
179 using SOAPdenovo-Trans-31mer with ‘-K 29 -L 100’. The programme Cd-hit (Li & Godzik  
180 2006), software for clustering and comparing protein or nucleotide sequences, was used to  
181 retrieve only unique contigs from the SOAPdenovo-trans analysis with the command cd-hit-est  
182 and default values. The output of Cd-hit was then fed into MarkerMiner v1.0 (Chamala *et al.*,  
183 2015) with parameters ‘-singleCopyReference Athaliana -minTranscriptLen 900’. MarkerMiner  
184 identifies and aligns putative orthologous single or low copy nuclear genes in a set of  
185 transcriptome assemblies, using a reciprocal BLAST search against a reference database  
186 (Chamala *et al.*, 2015). The alignments of genes included for further analyses were visually  
187 checked with minimal editing and trimming either side of the sequence where missing sites  
188 accounted for more than half of all available taxa. In subsequent analyses data from the two  
189 *Microbiota* accessions (Table 2) were amalgamated to represent one sample in order to minimise  
190 the amount of missing data for that species.

191

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## 192 **Phylogenetic analyses**

193 Alignments of putative single copy loci from MarkerMiner v1.0 (Chamala *et al.*, 2015) were  
194 used to compile two sets of data, the first comprising individual genes, in which each locus is  
195 treated independently, and the second a concatenated dataset in which all chosen loci were  
196 combined into one ‘super locus’. First, we used three conventional methods, MP, ML and  
197 Bayesian Inference to infer phylogenetic trees based on the concatenated dataset: analyses based  
198 on such dataset could lead to species-tree mis-inference if there is sufficient conflict between  
199 gene trees, but these concatenation-based methods often recovers the same tree that other  
200 species-tree estimation methods recover (e.g. Wickett *et al.*, 2014) and is commonly done to  
201 compare to other species-tree estimation methods, e.g. MP-EST (Liu *et al.*, 2010), STAR (Liu *et al.*,  
202 2009a), ASTRAL (Mirarab *et al.*, 2014). Maximum parsimony analysis (MP) was performed  
203 using PAUP\*4.0b10 (Swofford 2003), with gaps treated as missing data and polymorphic states  
204 as uncertain. A ‘branch and bound’ search with MulTrees on was carried out for both datasets.  
205 Branch support was estimated via bootstrapping with 1000 bootstrap replicates using heuristic  
206 searches (Felsenstein, 1985). We also used RAxML v8 (Stamatakis 2014) to estimate a  
207 maximum likelihood (ML) tree and ML bootstrap values, by applying the parameters ‘-f a -m  
208 GTRGAMMA -p 12345 -x 12345 -# 1000’ where the GTRGAMMA model and 1000 bootstrap  
209 replicates were applied (see RAxML manual for detailed parameter settings). A Bayesian  
210 inference analysis (BI) was also performed using MrBayes v. 3.1.2 (Huelsenbeck & Ronquist  
211 2001; Ronquist & Huelsenbeck 2003) with the GTR+I+G model, which was selected using  
212 MrModelTest v. 2.3 (Nylander 2004) under the Akaike Information Criterion. The analysis was  
213 run for 2 million generations with four MCMC chains in two independent parallel analyses, with  
214 one tree sampled every 500 generations. The average standard deviation of split frequencies was  
215 0.00000 at the end of the run. TRACER v1.5 (Rambaut & Drummond 2009) was used to assess  
216 the quality of the MCMC simulations and suggested a high degree of convergence between runs.  
217 The effective sample size values (ESS), that is the number of effectively independent draws from  
218 the posterior, were >500 for all parameters, indicating that sufficient sampling occurred.

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219  
220 From the individual gene dataset, we constructed individual ML gene trees for each locus  
221 using the software RAxML v8 (Stamatakis 2014) applying the parameters as for the  
222 concatenated data set. The topology of each gene tree was then manually examined, looking in  
223 particular for well-supported alternative relationships that might indicate gene-tree conflict.  
224 Then, based on these gene trees, we generated a species tree based on the multispecies coalescent  
225 model in ASTRAL 5.6.1 (Mirarab *et al.*, 2014; Mayyari and Mirarab, 2016), which estimates  
226 species trees from unrooted gene trees, and maximizes the number of quartet trees shared  
227 between the gene trees and the species tree. ASTRAL-II estimates branch lengths for internal  
228 branches (not terminal branches) in coalescent units, and branch support values measure the  
229 support for a quadripartition (the four clusters around a branch) and not the bipartition, as is  
230 commonly done. The species tree was fully annotated using “-t 4” option, which calculates the  
231 measurements for each branch, including quartet support (q), total number of quartet trees in all  
232 the gene trees (f), and the local posterior probabilities (pp) for the main topology and the first,  
233 second alternatives, total number of quartets defined around each branch (QC), and the effective  
234 number of genes for the branch (EN).

235 Conservative pp scores cause some true positives to be overlooked in ASTRAL (Mayyari  
236 and Mirarab, 2016); moreover, average positive branch rates, which represent the proportion of  
237 the estimated species tree in which a certain branch is successfully recovered, may be lower in  
238 ASTRAL than in STAR and MP-EST (Liu *et al.*, 2015). Therefore, we also conducted STAR  
239 and MP-EST analyses based on gene trees to reduce the chance of missing any true positives,  
240 and to improve the average positive branch rates. Hence the rooted ‘best tree’ RAxML output for  
241 each gene plus bootstrap values for each gene tree using 1000 replicates was then uploaded to  
242 ‘The Species TRee Analysis Webserver’ STRAW (Shaw *et al.*, 2013) to estimate the species tree  
243 using STAR (Liu *et al.*, 2009a) and MP-EST (Liu *et al.*, 2010). Both programmes apply the  
244 multispecies coalescent model (Rannala & Yang 2003) to obtain estimates of the species tree  
245 from gene trees. STAR (Liu *et al.*, 2009a) uses the average ranks of coalescences, whereas

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246 MP-EST (Liu *et al.*, 2010) uses a pseudo-likelihood function of the species tree, and both of  
247 them generate bootstrap support values using nonparametric bootstrap techniques (Liu *et al.*,  
248 2009a, 2010). Both methods are based on summary statistics calculated across all gene trees,  
249 with the effect that a small number of genes that significantly deviate from the coalescent model  
250 will have relatively little effect on the ability to accurately infer the species tree.

251 Because there was some well-supported conflict among gene trees (see Results), we conducted  
252 two additional analyses to investigate this further. First, we applied MulRF (Chaudhary *et al.*,  
253 2013, 2015) to estimate the best species tree, i.e. the one that minimizes the overall  
254 Robinson-Foulds (RF) distance between each candidate species tree and the individual gene trees.  
255 This software is also able to calculate the MulRF score of a given tree topology, which is the RF  
256 distance between this given tree and all gene trees. In a soft polytomy where relationship among  
257 three clades are difficult to resolve, this function may be used to compare the compatibility of  
258 each of the three dichotomy candidate species trees with all gene trees.

259 Finally, we used the NeighborNet method implemented in SplitsTree 4.11.3 (Huson and  
260 Bryant, 2006) to reconstruct phylogenetic networks based on the concatenated alignment of all  
261 73 nuclear genes. For distance calculations, we excluded insertions/deletions (indels) and used  
262 the K2P model (Kimura, 1980). The relative robustness of the clades was estimated by  
263 performing 1000 bootstrap replicates, and a confidence network was generated with a 95%  
264 threshold (Huson and Bryant, 2006). This analysis can summarize how homoplasy that might  
265 include hybridization or incomplete lineage sorting might have affected the phylogenetic  
266 reconstruction.

267

### 268 **Molecular dating**

269 To investigate the impact of topological differences on the evolutionary divergence timescale in  
270 Cupressoideae, we conducted molecular dating analyses. We tried to adopt the eight fossil  
271 calibration points as in Mao *et al.* (2010), but only three of them could be used for the dating of  
272 our 20-taxon data set, whereas the remaining five could only be attached to apparently deeper

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273 nodes, relative to those in the phylogeny of Mao *et al.* (2010). As too few calibration points  
274 and/or assigning fossils to deeper nodes (due to sparse sampling) has been shown to bias the  
275 estimates of node ages (e.g. Linder *et al.*, 2005; Mao *et al.*, 2012; Wang & Mao, 2016), we  
276 adopted a hybrid strategy to reconstruct the evolutionary divergence timescale of Cupressoideae.  
277 Hence dating was carried out on our previous ptDNA dataset comprising nine ptDNA fragments  
278 from 84 species (Mao *et al.*, 2010), but with the relationship between the three main clades  
279 constrained to the topology from the current study based on transcriptome data (see below). The  
280 original ptDNA dataset comprising 92 accessions was slightly reduced by removing multiple  
281 accessions of six species, resulting in a final dataset of 86 accessions representing 84 species in  
282 Cupressoideae (referred to as ‘86-accession data set’ from here on). Three parallel molecular  
283 dating analyses were carried out, one constraining to the HCX(Cu,Ju) topology, another  
284 constraining to the Cu(HCX,Ju) topology, and the third was unconstrained, allowing it to retain  
285 the Ju(Cu,HCX) topology from Mao *et al.* (2010). We adopted eight calibration fossils from Mao  
286 *et al.* (2010), seven of which were used as minimum age constraints with uniform priors, and one  
287 was set as a fixed age constraint with a normal prior (see Table 1 in Mao *et al.*, 2010 for details).

288 BEAST version 1.8.0 (Drummond & Rambaut, 2007) was used to simultaneously estimate  
289 topology, substitution rates and node ages by employing a Bayesian MCMC chain. BEAST  
290 parameter settings, including fossil calibration settings, were all the same as in Mao *et al.* (2010),  
291 except that two independent MCMC analyses of 100 000 000 generations were conducted,  
292 sampled every 2 000 generations, with 20% burn-in. The program Tracer 1.5.1 (Rambaut &  
293 Drummond, 2007) was employed to check effective sample size, and the program TreeAnnotator  
294 1.8.0 (part of the BEAST 1.8.0 package) was used to summarize the output results. Finally, a tree  
295 with ages for each node and their 95% highest posterior density intervals (95%HPD), was  
296 displayed and formatted in FigTree 1.3.1 (Rambaut, 2008).

297

## 298 **Ancestral area reconstruction**

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299 We conducted an ancestral area reconstruction using the BioGeoBEARS packages as  
300 implemented in RASP 4.0 (Yu *et al.*, 2015). Four operational geographic areas (A: North  
301 America, B: Africa, C: Asia, D: Europe), were defined for our analyses, following those in Mao  
302 *et al.* (2010). A total of 400 trees, which were resampled from the output trees of the BEAST  
303 analysis, and the BEAST summary tree, were imported into RASP 4.0, along with the  
304 distribution information of each species. BioGeoBEARS allows the testing of six models  
305 (DIVALIKE, DIVALIKE+J, DEC, DEC+j, BAYAREALIKE and BAYAREALIKE+J) (Matzke  
306 2013, 2014). Of the six models, the DIVALIKE model (Ronquist, 1997) is an event-based  
307 approach that adopts a simple biogeographic model; it does not consider general area  
308 relationships or branch lengths of the input tree, and it applies different costs to vicariance,  
309 duplication, dispersal, and extinction to construct ancestral distributions (Ronquist, 1997; Yu *et*  
310 *al.*, 2015). The DEC model (Ree and Smith, 2008) allows dispersal, extinction, and cladogenesis  
311 as fundamental processes, accommodates differing dispersal probabilities among areas across  
312 different time-periods, and can integrate branch lengths, divergence times, and geological  
313 information (Ree and Smith, 2008; Yu *et al.*, 2015). In contrast to the former two models, which  
314 accept only bifurcating trees, the BAYAREALIKE model (Landis *et al.*, 2013) allows  
315 polytomies. It considers distribution area to be a ‘trait’ of a species, and hence reconstructs  
316 ancestral ‘traits’ using Bayesian inference; furthermore, it does not define the dispersal rate,  
317 constrain the maximum number of areas at each node, or exclude widespread and unlikely  
318 ancestral areas before analysis (Landis *et al.*, 2013; Yu *et al.*, 2015). The other three models with  
319 the ‘+J’ suffix (i.e., DIVALIKE+J, DEC+j, BAYAREALIKE+J) allow founder-event speciation,  
320 in contrast to the three original models (Matzke, 2014).

321 We conducted model testing and two models (the best and the second-best model, as given in  
322 the Results section) were employed to reconstruct the ancestral area for every node in the  
323 phylogeny based on 100 trees that were randomly selected from 400 BEAST trees. At most two  
324 areas were allowed for any node in any tree. An among-area dispersal probability matrix, which  
325 is the same as in Mao *et al.* (2012), was coded to define different dispersal probabilities in five

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326 time periods, 0-5 Mya, 5-30 Mya, 30-45 Mya, 45-70 Mya and 70-115 Mya. The ancestral area  
327 reconstruction results were optimized in the Treeview window of the RASP program.

328

## 329 **RESULTS**

330

### 331 **Phylogenetic analyses**

332

333 A total of 581 putative single or low copy genes were detected by MarkerMiner. About 50% of  
334 these were shared by five or fewer samples. To minimise the impact of missing data on the  
335 ability to confidently resolve phylogenetic relationships, we only included genes that did not  
336 have more than two ingroup and two outgroup taxa missing, for further phylogenetic analyses.  
337 This resulted in 73 putatively single or low copy genes yielding an alignment of 208,484 nuclear  
338 base pairs for 20 taxa (Supplementary Table S1). The DNA sequences have been deposited in  
339 NCBI GenBank (accession numbers shown in Table S2).

340 For six of the 73 genes chosen by MarkerMiner (Chamala *et al.*, 2015), a secondary transcript  
341 that passed the BLAST filtering process was reported for one of the included taxa  
342 (Supplementary Table S1). These secondary transcripts, defined as the one of the two from a  
343 given taxon that received a lower BLAST score, may represent splice isoforms, putative  
344 paralogs, or partially assembled transcripts (Chamala *et al.*, 2015). However, as removing the  
345 taxon from the particular alignment for which a secondary transcript was detected did not change  
346 the phylogenetic results in any way, for these species (data not shown), the transcript for each  
347 taxon with the higher BLAST score was included in all analyses.

348 In this matrix consisting of 73 genes, 183,022 (87.8%) characters were constant, 16,405  
349 (7.9%) were variable but parsimony-uninformative and 9,057 (4.3%) were  
350 parsimony-informative. The same topology was retrieved regardless of the bifurcate  
351 tree-building method used (including MP, ML, BI, MP-EST, STAR, ASTRAL-II, MulRF), with  
352 an HCX clade (comprising a monophyletic *Hesperocypris*, plus *Callitropsis* and



353 *Xanthocyparis*) as the sister group of a clade consisting of *Cupressus* (monophyletic) and  
354 *Juniperus* (also monophyletic) (Figs 1, 2). Branch support was high for the MP, ML and BI  
355 analyses of the concatenated data set and for the MP-EST, STAR and ASTRAL-II analyses  
356 using a coalescent approach. Individual trees produced by Bayesian analysis and the three  
357 coalescent approaches were identical. The MP tree topology differed from these in only one  
358 respect: here (*J. procera* (*J. indica*, *J. microsperma*)) was sister to (*J. flaccida*, *J. scopulorum*)  
359 (Supplementary Fig. S1), whereas in the other analyses *J. procera* was sister to ((*J. indica*, *J.*  
360 *microsperma*) (*J. flaccida*, *J. scopulorum*)) (Figs 1, 2). However, two branches concerning this  
361 relationship were weakly or moderately supported by the bootstrap analysis in the MP analysis  
362 (BS=55% and 76%, respectively, Fig. S1). The *Juniperus* clade was also the only ingroup clade  
363 where some of the internal relationships did not receive maximum branch support; this was true  
364 for all six analyses methods used (MP, BI, ML, MP-EST, STAR and ASTRAL-II; Figs 1, 2).

365 The HCX(Cu,Ju) topology of our species tree based on single nuclear genes conflicts with the  
366 Ju(Cu,HCX) topology based on the ptDNA data from Mao *et al.* (2010) (Supplementary Fig. S2).  
367 Quartet support analyses in ASTRAL-II suggest that the HCX(Cu,Ju), Ju(Cu,HCX) and  
368 (Cu(HCX,Ju) topologies are supported by 54.16%, 24.24% and 21.60% of the gene trees,  
369 respectively (Fig. 2). A manual check of gene trees (Fig. S3) which were generated in RAXML  
370 using maximum likelihood bootstrapping (MLBS) indicated similar proportions of gene trees  
371 supporting these three topologies (i.e., most supporting the HCX(Cu,Ju) topology, which is  
372 equivalent to the *Cupressus-Juniperus* sister topology in Table S1), except that a few MLBS  
373 gene trees are unresolved (Table S3). We also calculated the MulRF score (the total  
374 Robinson-Foulds topological distance of all 73 gene trees against the candidate species tree) for  
375 each of the above three topologies concerning *Cupressus*, *Juniperus* and HCX (other  
376 relationships remain the same). The HCX(Cu,Ju), Ju(Cu,HCX) and Cu(HCX,Ju) topologies  
377 received MulRF scores of 744 (= closest and therefore favored), 786 and 790 (= furthest),  
378 respectively.

379 Finally, NeighborNet analyses provide 100% bootstrap support for the quartet branch that  
380 links (*Juniperus*, *Cupressus*) and (HCX, outgroups), although the length of this branch is  
381 relatively short (Fig. 3A). Very few strongly supported relationships that might have suggested  
382 hybridization or incomplete lineage sorting (bootstrap support >95%) were recovered in the  
383 NeighborNet confidence network; these were mainly found within *Juniperus*, but also once  
384 within *Cupressus* (the branch leading to (*C. gigantea*, *C. duclouxiana*)), at the basal position of  
385 the HCX clade, and among the three outgroups (Fig. 3B).

386

### 387 **Molecular dating**

388 The BEAST analysis based on the two phylogenetic topologies, HCX(Cu,Ju) and Ju(Cu,HCX),  
389 yielded effective sample sizes that were well above 200 (> 800) for branch lengths, topology and  
390 clade posteriors and all other relevant parameters, indicating adequate sampling of the posterior  
391 distribution. However, the BEAST analysis based on the Cu(HCX,Ju) topology failed, despite  
392 being identical to other analyses in all but enforcement of topology, because every one of >20  
393 attempts returned an error message that the log likelihood of the initial tree is negative Infinity.

394 Based on the HCX(Cu,Ju) topology that was supported by single-copy nuclear (SCN) genes,  
395 we estimate that the most recent common ancestor (MRCA) of *Cupressus*, *Juniperus* and the  
396 HCX clade diverged from the MRCA of *Platycladus*, *Microbiota*, *Calocedrus* and *Tetraclinis*  
397 81.06 Mya [70.50-90.40](from here on, shown in square brackets are the 95% HPD range of age  
398 estimation), the HCX clade diverged from the MRCA of *Cupressus* and *Juniperus* 59.80 Mya  
399 [48.45-71.74], and *Juniperus* diverged from *Cupressus* 56.33 [45.30-67.95] Mya. The crown  
400 ages of the HCX clade, *Cupressus* and *Juniperus* were estimated to be 37.45 Mya [23.54-52.30],  
401 28.73 Mya [16.65-42.15], and 41.34 Mya [29.99-44.63], respectively (Fig. 4, Table 3).

402 Based on the Ju(Cu,HCX) topology that was supported by ptDNA tree, age estimation for all  
403 nodes overlapped with the above estimation (see Table 3) except that the HCX clade diverged  
404 from *Cupressus* 54.09 Mya (95% HPD: 41.29-67.03). A comparison of age estimation of major  
405 nodes in BEAST analyses based on each of the above two topologies are shown in Table 3.

406

407 **Ancestral area reconstruction**

408 Model tests in the BioGeoBEARS package, based on either the HCX(Cu,Ju) or the Ju(Cu,HCX)  
409 topology, suggested that DIVALIKE+J is the best-performing model (AICc\_wt values:  
410 HCX(Cu,Ju) topology = 0.65, Ju(Cu,HCX) topology = 0.60), whereas DEC+J model is the  
411 second-best model (AICc\_wt values: HCX(Cu,Ju) topology = 0.33, Ju(Cu,HCX) topology =  
412 0.39).

413 Based on the HCX(Cu,Ju) topology, the DIVALIKE+J model and the 86-accession data set,  
414 *Cupressus*, *Juniperus* and the HCX clade share a common ancestor whose ancestral distribution  
415 area is probably Asia (ca. 0.96), whereas *Cupressus* and *Juniperus* shared a common ancestor  
416 whose ancestral distribution area is likely to be Asia (ca. 0.82) or less likely Europe (ca. 0.16).  
417 The ancestral area for the MRCA of the HCX clade is inferred to be Asia (ca. 0.54), North  
418 America (ca. 0.33) or a combination of these two (ca. 0.13). Within this clade, the common  
419 ancestor of all New World cypresses (*Callitropsis* plus *Hesperocyparis*) most likely migrated to  
420 and diversified in North America later (Fig. 5). The ancestral area for *Cupressus* is probably Asia  
421 (ca. 0.99), and *Cupressus semperivens* and its close allies dispersed to Europe around the middle  
422 Miocene (Fig. 5). Furthermore, the ancestral area for *Juniperus* is inferred to be Europe (ca.  
423 0.72), or possibly Asia (ca. 0.23). The common ancestor of sect. *Juniperus* was inferred to be in  
424 Europe (ca. 0.56), Asia (ca. 0.36) or a combination of both, whereas that of sect. *Sabina* was  
425 probably in Asia (ca. 0.65) and possibly in Europe (ca. 0.25) or Africa (ca. 0.05); overall,  
426 *Juniperus* is most likely to have diversified within Eurasia, with three separate dispersal events  
427 to North America, and one to Africa. BioGeoBEARS analysis based on the DEC+J model  
428 yielded highly similar results (not shown), especially concerning major nodes in the phylogeny.

429 Based on the Ju(Cu,HCX) topology, either the DIVALIKE+J or the DEC+J model and the  
430 86-accession data set, the ancestral area for nearly all nodes are highly similar to the HCX(Cu,Ju)  
431 topology, except for the node of the common ancestor of *Cupressus* and the HCX clade, which

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432 does not exist in the HCX(Cu,Ju) topology. The ancestral area for this node in the Ju(Cu,HCX)  
433 topology is likely to be Asia (DIVALIKE+J: ca. 0.97; DEC+J: 0.95) (Supplementary Fig. S4).

434

## 435 **DISCUSSION**

436

### 437 **Rapid evolutionary divergence and inference of phylogenetic relationships among the three** 438 **major clades in Cupressoideae**

439 The main aim of this paper is to resolve and explain the long-standing controversy of generic  
440 and inter-generic relationships between the three major lineages in Cupressoideae, *Cupressus*,  
441 the *Hesperocyparis-Callitropsis-Xanthocyparis* (HCX) clade, and *Juniperus*. Our phylogenetic  
442 analyses using maximum parsimony (MP), maximum likelihood (ML), Bayesian inference (BI)  
443 analyses of concatenated data and species tree analyses (MP-EST, STAR, and ASTRAL), based  
444 on 73 putative single copy nuclear genes totaling more than 200,000 base pairs, all show a  
445 maximally supported sister relationship of *Cupressus* and *Juniperus*, and that their common  
446 ancestor is sister to the HCX clade (Figs 1, 2). Although only weakly or moderately supported,  
447 the Ju(Cu,HCX) topology based on ptDNA (Supplementary Fig. S2; Mao *et al.*, 2010) conflicts  
448 with the HCX(Cu,Ju) topology here, as well as several published phylogenies (Xiang & Li, 2005;  
449 Little *et al.*, 2006; Adams *et al.*, 2009; Yang *et al.*, 2012; Terry & Adams, 2015). This  
450 incongruence may have been caused by incomplete lineage sorting due to rapid evolutionary  
451 divergence and/or hybridization and introgression between the three clades during their early  
452 evolutionary history. Yang *et al.* (2012) constructed a reticulate network using two nuclear loci,  
453 and because relationships of the three subclades were incongruent among different datasets,  
454 suggested that *Cupressus* “might have originated through hybridization between *Juniperus* and  
455 the ancestor of *Hesperocyparis-Callitropsis-Xanthocyparis*” (Yang *et al.*, 2012; p462). However,  
456 although hybridization and introgression during earlier history is a possibility, the main cause of  
457 the phylogenetic pattern among the three clades appears to be a combination of rapid  
458 evolutionary divergence and incomplete lineage sorting.

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459 First, all phylogenetic analyses we conducted based on 73 loci support the HCX(Cu,Ju)  
460 topology. As we have shown above, the species tree constructed using MP-EST, STAR,  
461 ASTRAL or trees built using MP, ML, BI based on concatenated data show 100% support for  
462 the HCX(Cu,Ju) topology. The species tree estimate from MulRF also supports the HCX(Cu,Ju)  
463 topology: in particular, the RF distance between all gene trees to the HCX(Cu,Ju) topology is  
464 closer than to either the Ju(Cu,HCX) topology or the Cu(HCX,Ju) topology. In addition,  
465 Neighbor-Net tree based on concatenated dataset also support the HCX(Cu,Ju) topology with  
466 100% bootstrap support (Fig. 3A), and the “reticulate” pattern among the three clades that Yang  
467 *et al.* (2012) reported was not detected (Fig. 3).

468 Second, the gene tree topology frequency we found here may fit better with incomplete  
469 lineage sorting as an explanation of conflicting gene trees. The maximum support value for  
470 nodes in the species tree does not necessarily mean that there is no conflict between the 73 gene  
471 trees. In our ASTRAL analyses, for example, the HCX(Cu,Ju), Ju(Cu,HCX) and Cu(HCX,Ju)  
472 topologies, received quartet support values of 54.16%, 24.24% and 21.60%, respectively  
473 (equivalent to 39.54, 17.69, 15.77 gene trees, respectively). We further checked the MLBS tree  
474 for each of the 73 genes and found that 38, 15, 14 gene trees support the above three topologies,  
475 respectively; if only MLBS values above 70% are considered (corresponding to a moderately  
476 well supported branch), then 31, 11 and 7 gene trees supported the three topologies, respectively.  
477 If conflict between gene trees is caused by incomplete lineage sorting, which is always a close  
478 companion of rapid evolutionary divergence (e.g., Maddison, 1997), then we would expect one  
479 high frequency topology and two lower frequency topologies. Conversely, if the conflict between  
480 gene trees is caused by hybridization and introgression (e.g. the hypothesis that *Cupressus* is a  
481 hybrid clade between *Juniperus* and HCX that Yang *et al.* (2012) have put forward), one might  
482 expect two major (and equivalent) frequency gene tree topologies (e.g., if the branch was a result  
483 of hybrid speciation) or some other set of frequencies (e.g., if a subset of the genome  
484 introgressed at this point). To conclude, the pattern of gene tree topology frequencies we found

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485 above is more consistent with the scenario of incomplete lineage sorting than the hybridization  
486 and introgression scenario.

487 Third, the internode branch lengths between the three clades are consistently short in both our  
488 species tree (Fig. 2) and trees based on concatenated data (Fig. 1), and the molecular dating  
489 suggest that the interval between the MRCA of HCX-*Juniperus-Cupressus* (~59.8 Mya) and the  
490 MRCA of *Juniperus-Cupressus* (~56.3 Mya) is also relatively short (~3.5 Myr), consistent with  
491 rapid evolutionary divergence (and presumably a substantial chance of retaining some  
492 conflicting ancestral polymorphisms, as documented for our individual gene trees, Fig. S3). This  
493 has also been the case in previous phylogenies of Cupressoideae. For example, using the whole  
494 plastid genome the inferred internode branch length is short, regardless of whether the  
495 HCX(Cu,Ju) or Ju(Cu,HCX) topology is recovered (Qu *et al.*, 2017), and based on six ptDNA  
496 regions the phylogenetic relationship of these three clades remained unresolved (Mao *et al.*,  
497 2012). However, there is one exception to the pattern, which is that the internode branch length  
498 between the MRCA of the three clades and the MRCA of *Cupressus* and *Juniperus* is relatively  
499 long based on the nuclear gene NEEDLY (Yang *et al.*, 2012).

500 Thus, although we cannot exclude reticulate evolution in shaping the current phylogenetic  
501 pattern of the three clades within Cupressoideae, rapid evolutionary divergence better explains  
502 the pattern we found. This inference is different from another case in this subfamily where  
503 reticulate evolution is clearly indicated among *Thuja* species (Peng and Dan, 2008).

504

505 **Transcriptomic data provide strong support for a four-genus taxonomic treatment in**  
506 ***Cupressus* s.l.**

507

508 Previous studies suggested four possible phylogenetic topologies concerning these three clades.  
509 Phylogenetic analyses based on either three or six ptDNA markers show that these three clades  
510 are part of a trichotomy (Little *et al.*, 2006; Mao *et al.*, 2012), whereas nine ptDNA markers  
511 provide moderate support for the Ju(Cu,HCX) topology (Mao *et al.*, 2010). A recent study based

---

512 on whole plastid genomes supported the clustering of *Juniperus* and *Cupressus*, while a filtered  
513 dataset, which was meant to reduce or elucidate long branch artifacts, supported the clustering of  
514 *Cupressus* and the HCX clade (Qu *et al.*, 2017). The Cu(HCX,Ju) topology was supported by a  
515 series of studies: based on nrITS region alone (Xiang & Li, 2005), a combined dataset that  
516 included nrITS, two ptDNA markers and 56 morphological characters (Little *et al.*, 2004); a  
517 combined dataset that included one ptDNA region and three nuclear regions (nrITS, ABI3, 4CL)  
518 (Adams *et al.*, 2009); and a combined dataset that included 11 ptDNA regions and two nuclear  
519 regions (nrITS and NEEDLY) (Terry & Adams *et al.*, 2015). Phylogenetic analyses based on a  
520 single nuclear region, NEEDLY (MP support value: 100%), and a combined dataset that  
521 included three ptDNA regions, two nuclear regions (ITS, NEEDLY) and 88 organismal  
522 characters (MP support value: 100%; Little, 2006) supported a HCX(Cu,Ju) topology, in  
523 agreement with our SCN results (Fig. 1).

524 One important implication of our results is that *Cupressus* s.l. is paraphyletic, and should be  
525 divided into four genera (see Fig. 1 and Table 1 for a clarification of taxon names). Nearly all  
526 published molecular phylogenetic analyses support the monophyly of both *Cupressus* s.s. and the  
527 HCX clade, yet the sister relationship between them is rarely supported (Mao *et al.*, 2010).  
528 Hence, Little (2006) proposed to call the HCX clade *Callitropsis* s.l., where *Xanthocyparis* s.l.  
529 was merged into *Callitropsis* s.l., yet such a treatment is not universally accepted. Considering a  
530 proposal of Mill and Farjon (2006) to conserve the genus name *Xanthocyparis*, which was  
531 ratified by the International Botanical Congress in 2011 (Barrie, 2011), and that *Xanthocyparis*  
532 s.l. is not monophyletic, Adams *et al.* (2009) proposed to place all New World cypresses  
533 (*Cupressus* sensu Farjon species in North America) in the new genus *Hesperocyparis* and keep  
534 both *Xanthocyparis* s.s. and *Callitropsis* s.s. as monotypic genera. Our results support this,  
535 showing that both *Cupressus* s.l. and *Xanthocyparis* s.l. are paraphyletic, while each of  
536 *Cupressus*, the HCX clade, and *Hesperocyparis* is monophyletic. Hence, our nuclear-based  
537 results strongly support the division of *Cupressus* s.l. into four genera: *Cupressus*,  
538 *Hesperocyparis*, *Xanthocyparis* s.s. and *Callitropsis* s.s. (Adams *et al.*, 2009; Mao *et al.*, 2010)

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539 and rejects both the combination of these four genera under *Cupressus* s.l. (Christenhusz *et al.*,  
540 2011; Table 1) and the combination of *Xanthocyparis* s.s. and *Callitropsis* s.s. under either  
541 *Xanthocyparis* s.l. or *Callitropsis* sensu Little (2004). Our data, as well as many others (e.g.  
542 Little, 2006; Mao *et al.*, 2010), would also be consistent with combining *Xanthocyparis* s.s. and  
543 *Callitropsis* s.s. and *Hesperocyparis* under *Callitropsis* s.l., yet *Hesperocyparis* is  
544 morphologically distinct enough to deserve recognition as a distinct genus (Adams *et al.*, 2009).

545

546 **An updated evolutionary divergence timescale and biogeographic history of *Cupressus*, the**  
547 **HCX clade and *Juniperus***

548 Rerunning the molecular dating on the ptDNA dataset from Mao *et al.* (2010) while constraining  
549 it with the nuclear species tree (HCX(Cu,Ju)) topology of our results, suggests that the split  
550 between the *Cupressus-Juniperus* clade and the HCX clade occurred (48.45-) 59.80 (-71.74)  
551 Mya, with a split of the former clade into *Cupressus* and *Juniperus* happening only 3.47 Myr  
552 later, (45.30-) 56.33 (-67.95) Mya. Comparing this to the Ju(Cu,HCX) topology that was  
553 supported by ptDNA data (Mao *et al.*, 2010), the only difference is that *Juniperus* diverges first  
554 (47.54-) 59.44 (-71.24) Mya, followed by the divergence of *Cupressus* from HCX (41.29-) 54.09  
555 (-67.03) Mya, in the Ju(Cu,HCX) topology. All other nodes occur in both topologies and differ  
556 in age between topologies by no more than 1.04 Myr, a difference dwarfed by HPD error ranges  
557 (Fig. 4, Table 3). This indicates that, in our case, a single topological difference, even in the deep  
558 nodes in a phylogeny, had very limited effect on node age estimates. A possible reason for this  
559 may be that this particular topological difference did not alter the phylogenetic position of fossil  
560 calibration points, and barely affected the total length between any given node and the root of the  
561 tree (Sauquet *et al.*, 2012; Wang & Mao, 2016).

562 We also reran the ancestral area reconstruction analyses for both the HCX(Cu,Ju) and  
563 Ju(Cu,HCX) topologies using BioGeoBEARS, and four parallel analyses were conducted for  
564 each of the two topologies based on two different models (DIVALIKE+J, DEC+J). Apart from  
565 the MRCA of *Cupressus* and *Juniperus*, and the MRCA of *Cupressus* and the HCX clade, that



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566 are specific to the HCX(Cu,Ju) and Ju(Cu,HCX) topologies, respectively, the relative probability  
567 of the ancestral area for all other nodes in all four parallel analyses are highly similar. We  
568 therefore discuss the reconstructed biogeographic history of the HCX clade, *Cupressus* and  
569 *Juniperus* based on the HCX(Cu,Ju) topology and the best model (DIVALIKE+J model). Our  
570 ancestral area reconstruction (AAR) analysis inferred that both the MRCA of the HCX clade,  
571 *Cupressus* and *Juniperus*, and the MRCA of *Cupressus* and *Juniperus*, most likely originated in  
572 Asia. Likewise, the HCX clade most likely originated in Asia and then dispersed once to North  
573 America and diversified there (Fig. 5). This fits a pattern of directional migration from the  
574 northwest to the southeast in North America in New World Cypresses (*Callitropsis* and  
575 *Hesperocyparis*), which may have been caused by climate cooling and aridification in the latter  
576 half of the Cenozoic (Terry *et al.*, 2016). *Cupressus* probably originated in Asia, and then  
577 dispersed to Europe (and northern Africa) around the middle Miocene (Fig. 5). The genus  
578 *Juniperus* and sect. *Juniperus* most likely originated in Europe, whereas sect. *Sabina* originated  
579 in Asia; three independent migrations from Eurasia to North America and one migration from  
580 Eurasia to Africa were inferred (Fig. 5).

581 Comparing these results to the previous AAR analysis based on S-DIVA (Mao *et al.*, 2010),  
582 the AAR analysis based on BioGeoBEARS yielded a clearer resolution, especially concerning  
583 the ancestral area of the MRCA of the HCX clade, the MRCA of *Juniperus*, the MRCA of  
584 *Juniperus* sect. *Juniperus* and sect. *Caryocedrus*, the MRCA of *Juniperus* sect. *Juniperus*, and  
585 the MRCA of Clade I (*Juniperus pseudosabina* plus all Himalayan/Qinghai-Tibet Plateau  
586 species except *J. microsperma* and *J. gausenii*) and Clade II (serrate-leaved junipers of North  
587 America) (Mao *et al.*, 2010). BioGeoBEARS tends to infer a single area as the ancestral area,  
588 whereas the S-DIVA usually infers the combination of two disjunct areas as the ancestral area.  
589 The integration of dispersal probability among areas during different time periods in the past,  
590 and the use of a model test to seek the best-performing model are likely to have improved the  
591 resolution of AAR in BioGeoBEARS compared to S-DIVA.

592

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## 593 **Conclusion**

594 Phylogenetic relationships among *Cupressus*, *Hesperocyparis-Callitropsis-Xanthocyparis* (HCX)  
595 and *Juniperus* have been a contentious issue since the discovery of the Golden Vietnamese  
596 Cypress, *Xanthocyparis vietnamensis*. Our species tree based on 73 nuclear loci yielded 100%  
597 support for a (HCX, (*Cupressus*, *Juniperus*)) topology which is in agreement with previous  
598 phylogenies based on two nuclear loci (LEAFY and NEEDLY; Yang *et al.*, 2012) and a  
599 combined dataset including both morphological characters and molecular dataset (Little, 2006),  
600 but contradicts many others. This indicates that *Cupressus* s.l. (Christenhusz *et al.*, 2011; Table 1)  
601 is paraphyletic, and can be considered instead as two monophyletic genera, *Cupressus* (s.s.) and  
602 *Hesperocyparis*, and two monotypic genera, *Callitropsis* (s.s.) and *Xanthocyparis* (s.s.). Rapid  
603 evolutionary divergence and incomplete lineage sorting may have been the major cause for the  
604 minor conflicts observed among gene trees. Molecular dating based on the nuclear species tree  
605 (HCX(Cu,Ju)) topology suggests that the three clades underwent two evolutionary splits in a  
606 time period as short as ca. 3.47 Myr. The split between *Cupressus+Juniperus* and the HCX  
607 occurred ca. 59.80 Mya (95%HPD: 48.45-71.74 Mya), and the split between *Cupressus* and  
608 *Juniperus* occurred ca. 56.33 Mya (95%HPD: 45.30-67.95 Mya). Ancestral area reconstruction  
609 analyses suggest that the MRCA of *Juniperus* probably occurred in Europe, whereas the MRCAs  
610 of HCX, *Cupressus*, *Cupressus+Juniperus*, and HCX+*Cupressus+Juniperus* all most likely  
611 occurred in Asia. Therefore, the common ancestor of these three clades most likely originated in  
612 Asia and then diversified and dispersed to Europe, North America and Africa. Our study shows  
613 that combining low copy nuclear genes collected using next generation sequencing and  
614 coalescent-based species tree estimation methods is a powerful approach that provides more  
615 refined phylogenetic estimates of deep nodes in conifer phylogeny that were controversial based  
616 on small datasets.

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625

## 626 **Author contributions**

627 K.M., M.R., J.L. designed research; K.M., M.R., Y.M., S.G., J.L., P.T., R.I.M., and P.M.H.  
628 performed research; M.R., K.M., Y.M. analyzed data; K.M., M.R., S.G., P.T., and P.M.H.  
629 procured specimens; K.M., M.R., R.I.M. wrote the manuscript, all authors revised the manuscript,  
630 and K.M., M.R., S.G., R.I.M. finalized the manuscript.

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- 788

789 **Tables**

790

791 **Table 1.** A brief summary of alternative taxonomic treatments of *Juniperus*, *Cupressus*,  
 792 *Hesperocyparis*, *Callitropsis* and *Xanthocyparis* since the description of *Xanthocyparis*  
 793 *vietnamensis* in 2002. Underlined taxa are either monophyletic or monotypic. The abbreviations  
 794 in brackets after common names are in accordance with Fig. 1.

(A)	Common names	Junipers	Old world cypresses (OWC)	New world cypresses (NWC)	Alaska cedar (A.)	Vietnamese golden cypress (V.)
(B)	This study; Adams <i>et al.</i> (2009); Mao <i>et al.</i> (2010, 2012)	<u><i>Juniperus</i></u>	<u><i>Cupressus</i></u> (s.s.)	<u><i>Hesperocyparis</i></u>	<u><i>Callitropsis</i></u> (s.s.)	<u><i>Xanthocyparis</i></u> (s.s.)
(C)	Farjon <i>et al.</i> (2002); Farjon (2005)	<u><i>Juniperus</i></u>	<i>Cupressus</i> sensu Farjon		<i>Xanthocyparis</i> s. l.	
(D)	Little <i>et al.</i> (2004)	N/A	<i>Cupressus</i> sensu Farjon		<i>Callitropsis</i> sensu Little (2004)	
(E)	Little (2006)	N/A	<u><i>Cupressus</i></u> (s.s.)	<u><i>Callitropsis</i></u> s.l.		
(F)	Christenhusz <i>et al.</i> (2011)	<u><i>Juniperus</i></u>	<i>Cupressus</i> s.l.			

795

796

797 **Table 2.** Accessions used for RNA extraction, transcriptome assembly and subsequent  
 798 phylogenetic analyses. (RBGE) and (SZ) refer to material collected from the wild held at the  
 799 Royal Botanic Garden Edinburgh and Sichuan University, respectively; (1kp) refers to  
 800 transcriptome data downloaded from the ‘1000 plant project’ ([http://www.onekp.com/samples/](http://www.onekp.com/samples/list.php)  
 801 [list.php](http://www.onekp.com/samples/list.php)) with vouchers held at the University of British Columbia (UBC).

802

Species	Collecting number (Identifier)	Lat/Long	Country
<i>Callitropsis nootkatensis</i>	19941704B (RBGE)	49°24'N/123°11'W	Canada
<i>Calocedrus decurrens</i>	FRPM (1kp)	n/a	cultivated (UBC)
<i>Cupressus duclouxiana</i>	MSZ-49-01 (SZ)	27°00'N/100°14'E	China
<i>C. funebris</i>	Mao-CF (SZ)	n/a	cultivated (SZ)
<i>C. gigantea</i>	MSZ-24-03 (SZ)	29°00'N/93°14'E	China
<i>C. sempervirens</i>	19752308 (RBGE)	45°12'N/13°36'E	Croatia
<i>Hesperocyparis arizonica</i>	19921324*C (RBGE)	30°50'N/115°16'W	Mexico
<i>H. bakeri</i>	19851378*B (RBGE)	41°57'N/123°18'W	USA
<i>H. macrocarpa</i>	20090071 (RBGE)	36°31'N/121°56'W	USA
<i>Juniperus drupacea</i>	20100261 (RBGE)	37°55'N/36°34'E	Turkey
<i>J. flaccida</i>	19922158*C (RBGE)	25°17'N/100°26'W	Mexico
<i>J. indica</i>	19790193*A (RBGE)	27°13'N/88°02'E	India
<i>J. microsperma</i>	MSZ-11 (SZ)	29°37'N/96°20'E	China
<i>J. oxycedrus</i>	19921237A (RBGE)	37°54'N/2°52'W	Spain
<i>J. phoenicea</i>	19921233*A (RBGE)	37°54'N/2°52'W	Spain
<i>J. procera</i>	20080832*J (RBGE)	00°19'N/36°58'E	Kenya
<i>J. scopulorum</i>	20081601 (RBGE)	39°39'N/105°12'W	USA
<i>Microbiota decussata</i>	19881678*A (RBGE)	n/a	cultivated (RBGE)
<i>M. decussata</i>	XQSG (1kp)	n/a	cultivated (UBC)

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<i>Thuja plicata</i>	VFYZ (1kp)	n/a	cultivated (UBC)
<i>Xanthocyparis vietnamensis</i>	20030523 (RBGE)	23°06'N/105°01'E	Vietnam

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804

805 **Table 3.** Estimates for the divergence times for nodes within *Juniperus* and *Cupressus* (s.s.) and  
 806 the *Hesperocyparis-Callitropsis-Xanthocyparis* clade (HCX), based on the ptDNA data set of  
 807 Mao *et al.* (2010) using the constraint of the nuclear species tree topology from this study  
 808 (HCX(Cu,Ju) topology) or without any constraint (i.e. ptDNA tree topology, Ju(Cu,HCX)  
 809 topology) employing a relaxed molecular dating approach in BEAST.

810

Node No.	Description of Node:	HCX(Cu,Ju) topology	Ju(Cu,HCX) topology
1	Stem of the MRCA of the three clades	81.06 (70.45-90.40)	80.96 (71.07-89.75)
2	Crown of the MRCA of the three clades	59.80 (48.45-71.74)	59.44 (47.54-71.24)
3	Split between <i>Cupressus</i> and <i>Juniperus</i>	56.33 (45.30-67.95)	Equal to Node 2
4	Split between <i>Cupressus</i> and the HCX clade	Equal to Node 2	54.09 (41.29-67.03)
5	Crown of <i>Cupressus</i>	28.73 (11.65-42.15)	28.44 (16.57-42.03)
6	Crown of the HCX clade	37.45 (23.54-52.30)	36.41 (22.73-50.81)
7	Split between <i>Callitropsis</i> (s.s.) and <i>Hesperocyparis</i>	32.30 (19.40-45.86)	31.58 (19.13-44.84)
8	Crown of genus <i>Juniperus</i>	41.34 (33.90-49.45)	41.79 (33.91-50.80)
9	Split: sects. <i>Juniperus-Caryocedrus</i>	33.80 (19.68-45.58)	34.12 (20.28-46.87)
10	Crown of sect. <i>Juniperus</i>	17.20 (8.63-27.41)	17.16 (8.34-27.05)
11	Crown of sect. <i>Sabina</i>	36.50 (29.99-44.53)	36.80 (29.49-44.86)

811

812

## 813 Figure descriptions

814

815 **Figure 1.** Bayesian tree based on 73 concatenated nuclear genes (208.484 bp). Numbers or  
816 asterisks above branches are statistical support values for maximum parsimony/maximum  
817 likelihood/Bayesian inference analyses, respectively, with \* denoting maximum support in all  
818 three analyses. Colour and grey scale bars to the right of the cladogram illustrate (A) common  
819 names of all included taxa (OWC = Old World cypresses; NWC = New World cypresses; A. =  
820 Alaska cedar; V. = Vietnamese golden cypress; C. = *Callitropsis*; X. = *Xanthocyparis*; sl/s.l. =  
821 sensu lato; sL = sensu Little (2004)), taxonomic treatments adopted (B) in this study, Adams *et*  
822 *al.* (2009) and Mao *et al.* (2010, 2012), (C) by Farjon *et al.* (2002) and Farjon (2005), (D) by  
823 Little *et al.* (2004), (E) by Little (2006) and (F) by Christenhusz *et al.* (2011). Scale bar indicates  
824 the estimated number of mutations per site.

825

826 **Figure 2.** Species tree generated using ASTRAL-II based on 73 nuclear genes (208.484 bp).  
827 Numbers or asterisks above branches are branch support values for MP-EST, STAR and  
828 ASTRAL-II analyses, respectively, with asterisks denoting maximum support in all three  
829 analyses. ASTRAL-II measures branch lengths in coalescent units (scale bar shown corresponds  
830 to two coalescent unit) for internal branches and NOT terminal branches (branch lengths of  
831 terminal branches are therefore arbitrary and meaningless). The pie chart shows respective  
832 quartet support for the main topology, the first and the second alternative topology (as shown in  
833 the inset). Note that in the inset, the tree formulas in parentheses were presented in the sense of  
834 an unrooted quadripartition (four-taxon) tree, where the central piece of the tree is an internode  
835 branch between the two pairs of partitions. The first case shown (*(Cupressus, Juniperus)*),(HCX,  
836 outgroups)) is consistent with HCX being sister to (*Cupressus, Juniperus*) in an outgroup-rooted  
837 tree.

838

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839 **Figure 3.** Neighbour-Net networks based on 73 concatenated nuclear genes (208.484 bp) using  
840 SplitsTree. (A) A Neighbour-Net network with bootstrap support values, and (B) a consensus  
841 Neighbour-Net network using a 95% threshold based on 1,000 bootstrap replicates. In (A),  
842 numbers next to “branches” are bootstrap support values. Note that in both (A) and (B) the  
843 branches lead to the three outgroup taxa are truncated so as to show more details of ingroup  
844 relationships.

845  
846 **Figure 4.** Evolutionary divergence timescale of Cupressoideae based on the ptDNA data set (86  
847 accessions from Mao *et al.*, 2010) with the imposed constraint of the nuclear species tree  
848 (HCX(Cu,Ju)) topology from this study using BEAST. Blue bars represent 95% HPD (highest  
849 posterior density) for each node, and white triangular with black outline represent compressed  
850 clades. Letters in black circles represent fossil calibration points (corresponding to those in Table  
851 1 in Mao *et al.*, 2010), and numbers in black squares indicate numbers for nodes of interest (see  
852 Table 3). ‘HCX’ stands for the *Hesperocyparis-Callitropsis-Xanthocyparis* clade.

853  
854 **Figure 5.** Ancestral area reconstruction based on BEAST trees constrained using nuclear species  
855 tree (HCX(Cu,Ju)) topology and the DIVALIKE+J model in BioGeoBEARS, as implemented in  
856 RASP 4.0. The inset shows the division of the distribution area of the five genera into four  
857 operational areas (North America, Africa, Asia and Europe). The pie at each node represents the  
858 reconstructed ancestral area; different colours of circular sector in a pie represent the relative  
859 probabilities of different ancestral areas at a node.

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## 862 **Supporting Information**

863

864 **Supplementary Table S1.** Detailed information for the 73 single/low copy nuclear genes used in  
865 phylogenetic analyses. The 'missing taxa' column highlights which taxa were not included due to  
866 missing data for a particular gene. Missing taxa in bold are from the ingroup; a list of acronyms  
867 and corresponding species names is given below the table.

868

869 **Supplementary Table S2.** NCBI GenBank accession numbers for the 73 genes of 20 taxa that  
870 were used in phylogenetic analyses.

871

872 **Supplementary Table S3.** A summary of the topology and the maximum likelihood (ML)  
873 bootstrap support value (MLBS) for each of the 73 nuclear genes used to construct the species  
874 tree. Different topologies showing the sister relationship between the three major clades,  
875 *Juniperus*, *Cupressus* and HCX, are shown. 'Other topology' refers to a situation where two or all  
876 of the three groups are polyphyletic and the topology does not fall into any of the first three  
877 categories. 'Polyphyletic group(s)' refers to a situation where one or more taxa are polyphyletic.  
878 'MLBS value' refers to the ML bootstrap support value for the sister relationship between the  
879 indicated clade.

880

881 **Supplementary Figure S1.** Maximum parsimony (MP) tree based on 73 concatenated nuclear  
882 genes (208,484 bp). MP analysis resulted in one tree of 31,272 steps with CI = 0.86 and RI =  
883 0.82. Scale bar indicates number of nucleotide changes. Numbers above or below branches are  
884 bootstrap support values based on 1000 bootstrap replicates.

885

886 **Supplementary Figure S2.** Phylogenetic relationships and posterior probability of major clades  
887 in Cupressoideae that were derived from BEAST using nine ptDNA fragments (86 accessions



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888 from Mao *et al.*, 2010) and eight fossil calibrations (a) without any constraint (i.e. ptDNA tree,  
889 Ju(Cu,HCX) topology) and (b) with a constraint using the nuclear species tree (HCX(Cu,Ju))  
890 topology concerning the relationship of *Cupressus*, *Juniperus* and the HCX clade (only) that was  
891 obtained in this study (see Fig. 1). Triangle tips represent clades that comprising more than two  
892 species; for full list of species see Figs 5 and S4, respectively. Posterior probabilities of clades  
893 shown to the right or upper-left of each node.

894

895 **Supplementary Figure S3.** Maximum likelihood bootstrap tree for each of the 73 nuclear genes  
896 as constructed in RaxML based on 1000 bootstrap replicates. Gene names are shown in the top  
897 left of each subfigures. Numbers close to each node represent the bootstrap support value for the  
898 branch lead to a node.

899

900 **Supplementary Figure S4.** Ancestral area reconstruction based on ptDNA tree (Ju(Cu,HCX))  
901 topology and the DIVALIKE+J model in BioGeoBEARS as implemented in RASP 4.0. The  
902 inset shows the division of the distribution area of the five genera into four operational areas,  
903 North America, Africa, Asia and Europe. The pie at each node represents the reconstructed  
904 ancestral area, different colours of circular sectors in a pie represent the relative probabilities of  
905 different ancestral areas at a node.

0.005









