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Diversification and biogeography of *Juniperus* (Cupressaceae): variable diversification rates and multiple intercontinental dispersals

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Summary

• A central aim of biogeography is to understand when and how modern patterns of species diversity and distribution developed. Many plant groups have disjunct distributions within the Northern Hemisphere, but among these very few have been studied that prefer warm semi-arid habitats.

• Here we examine the biogeography and diversification history of *Juniperus*, which occurs in semi-arid habitats through much of the Northern Hemisphere. A phylogeny was generated based on > 10 000 bp of cpDNA for 51 *Juniperus* species plus many outgroups. Phylogenies based on fewer species were also constructed based on nuclear internal transcribed spacer (nrITS) and combined nrITS/cpDNA data sets to check for congruence. Divergence time-scales and ancestral distributions were further inferred.

• Both long dispersal and migration across land bridges probably contributed to the modern range of *Juniperus*, while long-term climatic changes and the uplift of the Qinghai-Tibetan plateau probably drove its diversification. Diversification apparently slowed down during climate-stable period of the Oligocene, and then speeded up from the Miocene onwards.

• *Juniperus* probably originated in Eurasia, and was a part of the south Eurasian Tethyan vegetation of the Eocene to Oligocene. It reached America once at this time, once in the Miocene and once more recently.

Introduction

A central aim of biogeography is to understand when and how modern patterns of species diversity have developed, and how individual taxa reached their current locations (Donoghue *et al.*, 2001; Milne & Abbott, 2002). Intercontinental disjunctions within the Northern Hemisphere occur in hundreds of plant genera, sometimes accompanied by diversification events in one or more regions (Wolfe, 1975; McKenna, 1983; Tiffney, 1985a,b; Woodburne & Swisher, 1995; Xiang *et al.*, 1998, 2000, 2004, 2005; Wen, 1999; Tiffney & Manchester, 2001; Milne & Abbott, 2002; Donoghue & Smith, 2004; Feng *et al.*, 2005; Milne, 2006; Nie *et al.*, 2006a, b, 2008; Mansion *et al.*, 2008). Two intercontinental land bridges, that is, the North Atlantic Land Bridge (NALB) and Bering Land Bridge (BLB), are critical to understanding these floristic disjunctions (Wolfe, 1975; Tiffney, 1985a; Wen, 1999; Tiffney & Manchester, 2001; Milne, 2006; and references within them). The NALB was present during the early Tertiary but gradually broke up between 50 and 15 million years ago (Mya), making plant migrations progressively more difficult, although island chains may have permitted migration for some time after the direct land connection had gone (Tiffney, 1985a, 2000; Milne & Abbott, 2002). The BLB, by contrast, was present for most of the Tertiary, until

5.5–5.4 Mya (Marinkovich *et al.*, 1990; Tiffney, 2000; Gladenkov *et al.*, 2002), although local climatic cooling (Wolfe, 1994; White *et al.*, 1997; Tiffney & Manchester, 2001) probably cut off this migration route for many taxa before this (Milne & Abbott, 2002; Milne, 2006). Long-distance dispersal remains an alternative hypothesis for any disjunctions (Renner, 2004; Milne, 2006), although fossil and other evidence tends to favour vicariance for many Northern Hemisphere disjunctions (Milne & Abbott, 2002). Dispersal tends to be invoked for Northern Hemisphere disjunctions only when no land migration route existed at the time of migration (e.g. Coleman *et al.*, 2003; Wang *et al.*, 2007), especially in a few specific regions (for example, the Arctic: Abbott & Brochmann, 2003; Brochmann & Brysting, 2008).

Most work on Northern Hemisphere disjunctions has centred on eastern Asia–North America disjunct groups, which tend to be distributed in areas of moderate to high rainfall (Tiffney, 1985b; Xiang *et al.*, 1998, 2000; Wen, 1999; Donoghue *et al.*, 2001; Milne & Abbott, 2002; Donoghue & Smith, 2004; Milne, 2006). In contrast, there has been less work on disjunctions involving genera of semi-arid habitats, which tend to be Mediterranean–North American (e.g. Liston *et al.*, 1989, 1992; Liston, 1997; Hileman *et al.*, 2001; Coleman *et al.*, 2003; Hohmann *et al.*, 2006) or central Asian–Mediterranean disjunctions (e.g. Sun & Li, 2003). These disjunctions might be remnants of belts of evergreen vegetation adapted to semi-arid habitats similar to those of the modern Mediterranean, which existed at low latitudes on both the American (Madrean) and Eurasian (Tethyan) sides of the widening Atlantic during the middle Tertiary (Engler, 1879; Thorne, 1972; Axelrod, 1975; Wen & Ickert-Bond, 2009; Fig. 1b). These belts might have been connected via the NALB (Milne & Abbott, 2002), or possibly the BLB (Stebbins & Day, 1967; Hohmann *et al.*, 2006; Wen & Ickert-Bond, 2009). Alternatively, floristic similarities could reflect dispersals across a then narrower Atlantic (Raven, 1972; Raven & Axelrod, 1975; Shaw *et al.*, 2003), possibly via an intervening island chain (Axelrod, 1975; Liston *et al.*, 1989; Hileman *et al.*, 2001), although hard evidence for such a chain is lacking (Milne & Abbott, 2002).

The genus *Juniperus* is a major component of arid and semi-arid tree/shrub ecosystems throughout the Northern Hemisphere (Thorne, 1972; Adams, 2004, 2008a; Farjon, 2005), and is therefore an ideal model for examining the origins of disjunctions among arid northern floras. The genus is monophyletic (Adams, 2004, 2008a; Little, 2006), and Adams (2004, 2008a) recognized three monophyletic sections: *Caryocedrus*, with one species in the Mediterranean; sect. *Juniperus*, with nine species in East Asia and the Mediterranean plus the circumboreal *Juniperus communis*

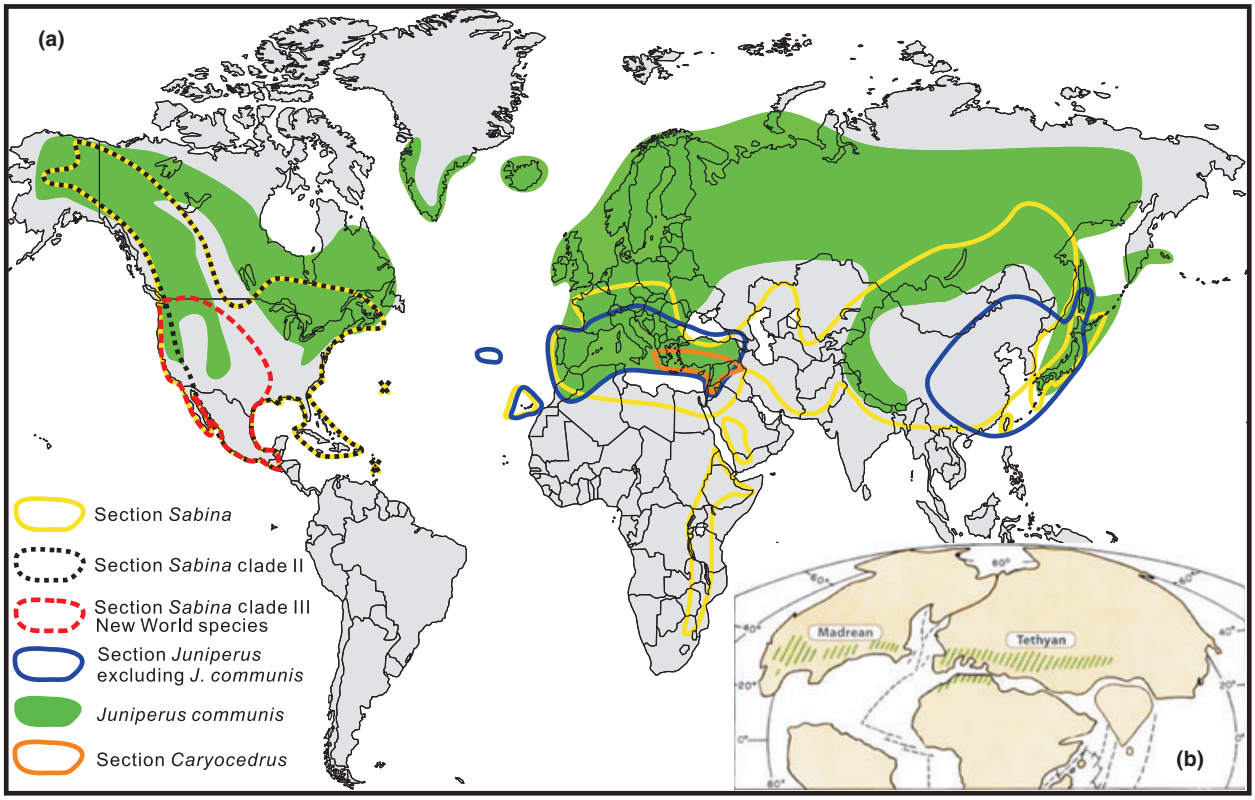

Fig. 1 The distribution of *Juniperus* (a, see Adams, 2008a) and the hypothesized Madrean-Tethyan vegetation belt (b, redrawn from Wen & Ickert-Bond, 2009; modified from Axelrod, 1975).

(ten in total); and sect. *Sabina*, with 56 species distributed in southwestern North America, Asia and the Mediterranean region, with outliers in Africa and the Canary Islands (Fig. 1).

Fossil records for sect. *Sabina* date from the Eocene/Oligocene boundary (Kvaček, 2002) in Europe, and the late Oligocene to early Miocene in North America (Axelrod, 1956, 1987, 1991; Wolfe, 1964), which is consistent with the hypothesis that *Juniperus* had become a component of Madrean-Tethyan vegetation belts on either side of the Atlantic (Axelrod, 1975) by the late Oligocene. It therefore must have somehow dispersed from one side to the other before this time. However, sect. *Sabina* did not certainly reach Asia until the late Pliocene (Dorofeev, 1962). Sects *Juniperus* and *Caryocedrus* are not known from the fossil record in North America or Asia, and only appear in Europe from the middle Miocene onwards (Straus, 1952; Negru, 1972; Bůžek *et al.*, 1985) and the Pliocene (Rérolle, 1884; Marty, 1903; Lauby, 1910; Palamarev, 1989), respectively.

Previous phylogenetic examinations of *Juniperus*, while highly informative, involved less than half of all extant species (Xiang & Li, 2005; Little, 2006; Adams, 2008a), and did not include molecular dating. Hence the routes and timings of inter-continental migrations within this genus remain obscure. In this study, we constructed phylogenetic relationships among three-quarters of extant *Juniperus* species; that is, 51 of the 67 recognized by Adams (2008a). We combined data from nine cpDNA markers (totalling > 10 000 bp), and dated divergence events using relaxed molecular clock approaches. We further tested this phylogeny against one based on independent nuclear internal transcribed spacer (nrITS) data, to check for congruence. In this study, we aimed to examine the diversification history of *Juniperus* and relate this to paleoclimates and paleogeography, reconstruct the past biogeography of *Juniperus*, including its area of origin and the likely causes of subsequent intercontinental migrations, and test the hypothesis that *Juniperus* originated as part of the Madrean-Tethyan floristic community.

Materials and Methods

A total of 116 accessions were examined for this study. Within *Juniperus*, 77 accessions were examined, representing 51 (out of 67; Adams, 2008a) extant species. Multiple accessions were included for *Juniperus* species that are widespread (e.g. *J. communis*) or whose classification is disputed, such as species from the Qinghai-Tibetan Plateau (QTP; Farjon, 2005; Adams, 2008a; Opgenoorth *et al.*, 2010). In addition, because wide taxonomic sampling permitted the use of fossil calibration points outside of *Juniperus*, we also included in the analysis a total of 38 species representing all of the other genera that comprise Cupressaceae *sensu stricto* (Gadek *et al.*, 2000; Supporting Information Table S1). This included 24 accessions of 23 species within *Cupressus* sens. lat. (Xiang & Li, 2005), which previous analyses (Little *et al.*, 2004; Little, 2006) have indicated contains the closest sister group to *Juniperus*. *Cupressus* sens. lat. may be subdivided into four genera, that is, *Cupressus* sens. str. and *Xanthocyparis* (sens. str.) in the Old World, and *Hesperocyparis* and *Callitropsis* (sens. str.) in the New World (Farjon *et al.*, 2002; Little, 2006; Mill & Farjon, 2006; Adams *et al.*, 2009). However, for this paper we will treat *Cupressus* sens. lat. as a distinct entity for ease of discussion.

Where possible, silica gel-dried fresh material from wild or cultivated accessions was used for DNA extraction, but for 25 accessions only herbarium material from the Royal Botanic Gardens at Kew or Edinburgh was available (Table S1). For silica gel-dried and Edinburgh herbarium material, total DNA were extracted from 10 to 20 mg of silica gel-dried leaf material using DNAeasy (Qiagen, Valencia, California, USA) extraction kits or a modified CTAB extraction method (Doyle & Doyle, 1987); for samples from Kew, DNA was extracted and delivered to us by Kew staff following their protocol (http://data.kew.org/dnabank/introduction.html).

We selected nine cpDNA regions, that is, the commonly used *mat*K, *rbc*L and *trn*L-F regions plus six others identified as useful, that is, *rps*4, *trn*S-G, *trn*D-T, *trn*V, *pet*B-D and *psb*B_1-B_2 (Taberlet *et al.*, 1991; Souza-Chies *et al.*, 1997; Wang *et al.*, 1999; Kusumi *et al.*, 2000; Grivet *et al.*, 2001; Shaw *et al.*, 2005 and references therein). Initial primers were taken from other studies (Table S2), but for longer regions, additional internal primers were necessary for complete sequencing where DNA quality was poor; these were designed for the current study based upon preliminary results (Table S2). In addition, we compiled a matrix of nrITS sequences from 22 species representing all sections and major cpDNA lineages identified within *Juniperus*, plus two outgroups. nrITS sequences for *Juniperus procumbens*, *Juniperus sabina* (var. *vulgaris*), *Juniperus excelsa* and *Juniperus phoenicea* were generated for this study; all others were taken from GenBank.

All polymerase chain reactions (PCRs) were performed in 25-μl reaction mixture volumes using reagents and manufacturer's instructions for Taq polymerase (Takara, Dalian, China; VH Bio, Gateshead, UK). PCR cycling programmes were designed individually for each primer pair (Table S3). PCR purification kits provided by Promega (Madison, Wisconsin, USA), Qiagen or CAS Array (Shanghai, China) were used to purify PCR products. Sequencing reactions and successive purifications were performed and capillary analyses were run on either ABI 3130XL (Lanzhou University, Lanzhou, China) or ABI3730 (The Gene Pool, University of Edinburgh, Edinburgh, UK), following the manufacturers' protocols.

Sequence alignment, gap coding and phylogenetic analysis

The sequences produced were gathered and aligned using ClustalX version 1.83 (Thompson *et al.*, 1997), followed by manual adjustments in Mega4 (Tamura *et al.*, 2007). Sequences from all nine cpDNA regions were concatenated into a single matrix for all analyses, because common inheritance without recombination for cpDNA markers can be assumed. Including indels can improve support values in a phylogenetic analysis (Simmons *et al.*, 2001), so all indels detected were coded using the simple code method applied by the program GapCoder (Young & Healy, 2003), and included in the maximum parsimony (MP) analysis.

Phylogenetic trees were constructed using MP, maximum likelihood (ML) and Bayesian methods (Fig. 2). MP analysis was conducted using paup 4.10b (Swofford, 2002) on the freely available Oslo Bioportal (http://www.bioportal.uio.no). A heuristic search was employed, with a starting tree obtained via stepwise addition, one tree held at each step

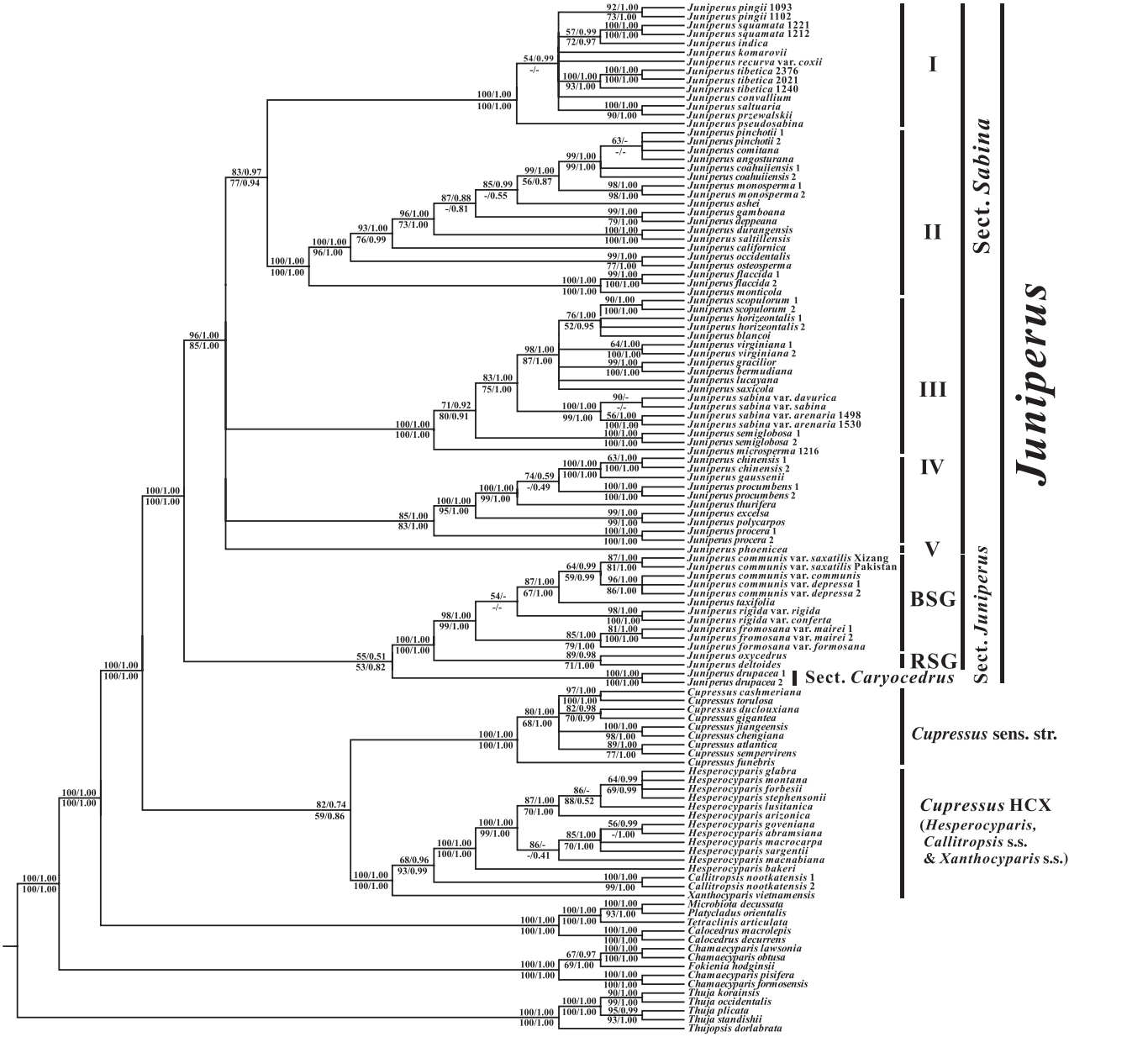

Fig. 2 Molecular phylogenetic relationships within *Juniperus*. Numbers above branches are maximum parsimony bootstrap support values (before slashes) and Bayesian posterior possibilities (after slashes), while numbers under branches are maximum likelihood bootstrap support values (before slashes) and beast posterior possibilities (after slashes).

during stepwise addition, tree bisection reconnection (TBR) branch swapping, steepest descent, MulTrees and Collapse options in effect, and no upper limit for the number of trees held in the memory. Support values for all nodes within the strict consensus tree were calculated via bootstrap analysis with the same settings as above and 1000 replicates; for each replicate, 10 searches with random taxon additions were conducted and the shortest tree was saved. ML analysis was implemented in GARLI version 0.96 beta (Zwickl, 2006) starting from random trees and using 5 000 000 generations per search; 30 searches were performed and the best tree saved. ML bootstrap analysis was carried out with the same program and settings, using 100 replicates and with five searches per replicate.

Before Bayesian analysis, the optimal model of molecular evolution was determined to be GTR + I + R ($-\log_e L$ = 22217.4902, K = 10, Akaike information criterion (AIC) = 44454.9805, base frequencies (A, C, G, T) = (0.3022, 0.1737, 0.1981, 0.3261), Nst = 6, rate matrix = (1.5357, 1.8436, 0.2916, 0.6853, 1.9191, 1.0000), proportion of invariable sites = 0.6166, gamma distribution shape parameter = 1.0273) by the AIC using MRMODELTEST version 2.3 (Posada & Crandall, 1998; Nylander, 2004; Posada & Buckley, 2004). Bayesian inferences were implemented in MRBAYES version 3.1.2 (Huelsenbeck & Ronquist, 2001) on Oslo Bioportal (http://www.bioportal.uio.no) with the model as above. One cold and three heated chains were started from random initial trees and run for 6 000 000 generations, with sampling every 200 generations. After a burn-in period of the first 20 000 generations, 20 000 trees were sampled from the posterior distribution, and a majority rule consensus of these was generated to provide posterior probability scores for all nodes.

In addition to these phylogenetic analyses of 116 taxa based on cpDNA data, we further constructed MP trees using the same settings for the 24 species for which we had nrITS data, based on nrITS data alone, and combined nrITS and cpDNA data (gaps were coded as above). For the latter, we tested for incongruence between the nrITS and cpDNA data sets using the 'partition homogeneity' test implemented in PAUP, with 100 replicates.

Origin of lineages through time

A lineage through time (LTT) plot was generated in R 2.9.0 (R Development Core Team, 2009) with 'laser' (Rabosky, 2006), 'geiger' (Harmon *et al.*, 2008) and 'ape' (Paradis *et al.*, 2004) packages loaded. The BEAST chronogram was used to produce an LTT plot for this genus, based on mean node age only, by applying the 'ltt.plot' command line.

A Cramer–von Mises test (Stephens, 1974; Paradis, 1998; Paradis *et al.*, 2004) was employed to test if diversification of *Juniperus* was constant through time. Furthermore, diversification rates for each geological epoch, subepoch or period were generated with survival analysis (Paradis, 1997; Paradis *et al.*, 2004) by employing the command line 'rate.estimate' within the R package 'geiger' (Harmon *et al.*, 2008). A stepwise plot of diversification rate through time was produced within R 2.9.0 with the command line 'plot' (R Development Core Team, 2009).

Molecular dating and fossil calibrations

We tested the hypothesis that a molecular clock could be fitted to our data by applying a χ^2 test, between the $-\log_e L$ values of distance trees with Enforce Clock (EC) and Without Clock (WC) a strict molecular clock enforced (Felsenstein, 1981). Our data set strongly rejected a strict molecular clock (df = 91, $2\log_e L(\text{EC} - \text{WC}) = 261.16$, $P < 0.01$), indicating that there was strong rate heterogeneity among different lineages. Therefore, three different relaxed molecular clock methodologies were applied to generate an evolutionary time-scale for *Juniperus* and related genera: penalized likelihood rate smoothing (PLRS; implemented in R8S; Sanderson, 2002), MULTIDIVTIME (Thorne & Kishino, 2002) and BEAST (Drummond & Rambaut, 2007).

PLRS calculations were carried out using the R8S program according to the R8S manual (Sanderson, 2003). First, a cross-validation procedure was performed based on the ML tree and calibration constraints, and a smoothing value was determined. With this smoothing value a chronogram was generated via PLRS, employing the truncated Newton (TN) algorithm. To determine 95% confidence intervals for node ages, 100 bootstrap replicate versions of the original sequence data set were generated using SEQBOOT (a program within the PHYLIP 3.68 package: Felsenstein, 2004). From each replicate sequence, a tree was then generated in PAUP 4.10b with topology constrained to that of the ML tree, but allowing branch length to vary. Cross-validation and PLRS were then applied to each replicate tree and, from this, standard deviation and 95% confidence intervals for ages of each node were calculated and summarized using R8S-95%CI-BOOTKIT (T. Eriksson, available at http://www.bergianska.se/index_forskning_soft.html).

MULTIDIVTIME (Thorne & Kishino, 2002) calculations were performed following Rutschmann's (2005) step-by-step manual. BASEML from Yang's (1997, 2007) PAML package was employed to generate an ML tree and a series of parameters that related under the molecular evolution model F84 + G (Felsenstein, 1993); next, the PAML2MODELINF program (Thorne & Kishino, 2002) was employed to summarize model parameters from BASEML output files; then a ML tree with branch lengths and a variance-covariance matrix was generated using the program ESTBRANCHES (Thorne & Kishino, 2002). Afterwards, the congruence between ML trees derived using BASEML and ESTBRANCHES was checked by comparing their $-\log_e L$

values. Finally, we performed two parallel runs, each of 3 million Bayesian Markov chain Monte Carlo (MCMC) generations, sampling every 100 generations, with the first ten thousand discarded as burn-in, within MULTIDIVTIME to approximate the posterior distribution of divergence times. Through such two runs, prior distribution of root height (rttm = 100, rttmsd = 100) and root rate (rtrate = 0.1, rtratesd = 0.1) were set according to the preliminary calculations, while the uppermost limit of root height was set to 400.0 Mya according to the earliest known seed plant fossil. The ages of each node derived from these two runs were checked for congruence. Only when the results are very close to each other (± 0.5 Mya) did we deem them as credible, and output from the final run was used as the estimation of each node.

BEAST version 1.4.8 (Drummond & Rambaut, 2007) was used to simultaneously estimate topology, substitution rates and node ages employing a Bayesian MCMC chain. Under the GTR model of nucleotide substitution with a gamma distribution and four rate categories, the Yule process tree prior model was implemented with rate variation across branches assumed to be uncorrelated exponential and lognormally distributed (Drummond *et al.*, 2006). The rate variation model (relaxed clock: uncorrelated lognormal) that yielded higher posterior probability estimates was employed to perform the final analysis. For all analyses, posterior distributions of parameters were approximated using two independent MCMC analyses of 50 000 000 generations with 20% burn-in. The program TRACER 1.4.1 (Rambaut & Drummond, 2007) was used to check effective sample size and the program TREEANNOTATOR 1.4.8 (part of the BEAST 1.4.8 package) was used to combine all samples and converge and/or summarize the output results. Finally, a tree with ages for each node and their 95% credible intervals (i.e. 95% highest posterior density intervals in the BEAST manual) were displayed and modified in FIGTREE 1.2.3 (Rambaut, 2008).

We used a total of eight fossil calibration points, of which three were within *Juniperus* and five outside it. Each fossil was assigned to a node based on its morphology (Table 1; Fig. 3; Notes S1). In preliminary analyses using BEAST, meaningful results could not be obtained unless two fixed age calibration points were included. Therefore we fixed the age of the two oldest calibration points within the phylogeny, and used the six younger fossils as minimum age calibration points (Fig. 3; and see Table 1 and Notes S1 for additional fossil information).

Biogeographic reconstruction

Four operational geographic areas (A, Europe plus North Africa and northern Arabia; B, Asia; C, North America, including the Caribbean and Central America; D, eastern Africa plus southern Arabia; see Fig. 4) were defined for our analysis. Use of four areas was found to give the best results because preliminary reconstructions based on five areas (splitting Asia into middle and east Asia), or six (also splitting North America into western and eastern parts) generated numerous ambiguous results. The boundaries of the four areas were defined in part so as to minimize the number of species that fell within two areas; all species except the widespread *J. sabina* and *J. communis* occurred within one area only. The Mediterranean species (e.g. *J. excelsa*, *J. phoenicea*, *J. polycarpos*, *J. oxycedrus* and *J. deltoides* etc.) are all distributed exclusively in area A, indicating a natural division for the genus between areas A and B. Areas A and D as defined here were respectively north and south of the Tropic of Cancer, which runs along the middle of a broad belt of very low precipitation (< 100 mm yr^{-1}) stretching across all of North Africa and most of Arabia (Geelan & Lewis, 1992). Throughout its distribution, *Juniperus* avoids areas of < 100 mm rainfall, so this belt forms a significant biogeographic barrier to the genus.

The recently developed Bayes-DIVA approach (Nylander *et al.*, 2008) was employed to infer the likely ancestral areas (geographical locations) of nodes within the *Juniperus* phylogeny (cpDNA), and hence infer its likely area of origin and patterns of subsequent migration. This method accommodates phylogenetic uncertainty into biogeographic reconstruction by utilizing the posterior distribution of trees resulting from a BEAST analysis (Nylander *et al.*, 2008). The last 10 000 trees were extracted from the combined tree file of BEAST analysis and used to reconstruct ancestral areas using the program DIVA 1.2 (Ronquist, 1997). The maximum number of ancestral areas for each node was constrained to two (maxareas = 2), which is equivalent to assuming that ancestral ranges might have covered two continents but no more, and hence were not more widespread than those of their extant descendants (Sanmartín, 2003). Because DIVA only accepts one tree at a time, a set of Perl scripts (kindly provided by J. A. Nylander; http://www.abc.se/~nylander/bayesdiva/bayesdiva.html) was used to prepare input files and parse output files. Bayes-DIVA outputs probabilities that a node was located within each defined geographical area, or that it was simultaneously in two such areas, as noted above. For each node, these probabilities were based on the average of results for all trees in the sample, excluding any trees wherein that node was not present. When several equally parsimonious reconstructions at a given node (e.g. A/B/AB) were obtained, these were downweighted by $1/n$, where n is the total number of alternative reconstructions at the node. Based on summarized results, the frequency of ancestral areas for each clade was then plotted on the BEAST maximum clade credibility (MCC) tree. In addition to Bayes-DIVA analysis for cpDNA data, we also carried out DIVA analysis (maxareas = 2) for *Juniperus* based on combining (cpDNA + nrITS) MP phylogeny.

Table 1 Fossils used as calibration points for molecular dating, their ages and taxonomic assignments, and the nodes they were attached to

Label	Fossil name	Stratigraphic period	Age constraint (Mya)[1]	Morphology and taxonomic assignment	Node it was used to constrain	References
A	*Juniperus pauli*	Eocene/Oligocene boundary	≥ 33.9	This fossil contains a mixture of characters found in different clades of sect. *Sabina*	Stem lineage of sect. *Sabina*	Kvaček, 2002
B	*Juniperus creedensis*	Late Oligocene	≥ 23.0	Seed cones and shoots resembling the extant *J. osteosperma* and *J. californica*	Crown lineage of clade II, sect. *Sabina* (= stem lineage of the MRCA of *J. osteosperma* and *J. californica*)	Axelrod, 1987
C	*Juniperus desatoyana*	Early Miocene	≥ 16.0	Seed cones and twigs very similar to the extant *J. occidentalis,* but this could be the common ancestor of *J. occidentalis*, and the similar *J. osteosperma*	MRCA: *J. occedentalis* and *J. californica* (= stem lineage of the common ancestor of *J. occidentalis* and *J. osteosperma*)	Axelrod, 1991
D	*Calocedrus suleticensis*	Early Oligocene	≥ 28.4	The ovulate cone and seed morphology of this fossil taxon allows assignment with confidence to the extant genus	Stem lineage of *Calocedrus* (= MRCA for *Calocedrus* and *Platycladus*)	Kvaček, 1999
E	*Tetraclinis salicornioides*	Oligocene	≥ 23.0	Co-occurring foliage, seed or seed cone remains indicate that this belongs within the extant monotypic genus *Tetraclinis*	MRCA: *Tetraclinis* and *Platycladus* (= stem lineage of *Tetraclinis*)	Kvaček *et al.*, 2000
F	*Fokienia ravenscragensis*	Early Paleocene	≥ 61.7	Despite similarities to other genera, this fossil is closest to the extant monotypic genus *Fokienia*	MRCA: *Fokienia* and *Chamaecyparis obtusa* (= stem lineage of *Fokienia*)	McIver & Basinger, 1990; McIver, 1992
G	*Chamaecyparis corpulenta*	Cretaceous (Santonian)	= 83.5	This fossil closely resembles extant *Chamaecyparis* lineages, indicating that diversification in this genus had begun	Crown lineage of *Chamaecyparis*	McIver, 1994
H	*Cupressinocladus interruptus*	Late Cretaceous to Tertiary	= 99.6	Placed by morphology within *Chamaecyparis/Fokienia* clade (also includes extinct genera *Mesocyparis* and *Cupressinocladus*)	Stem lineage of *Chamaecyparis/Fokienia* clade[2]	Stockey *et al.*, 2005 and references therein[3]

[1]Conversion of stratigraphical divisions into age ranges followed Grandstein *et al.* (2004) and ICS 2007 (*International Stratigraphic Chart* by International Commission on Stratigraphy, available at http://www.geobiodiversity.com/Download/ISC.pdf).
[2]*Chamaecyparis* is paraphyletic in our analysis, so this node is older than that for calibration point F.
[3]Florin (1963); Schweitzer (1974); McIver & Basinger (1987, 1990); McIver (1992, 1994); McIver & Aulenback (1994); Serbet (1997).

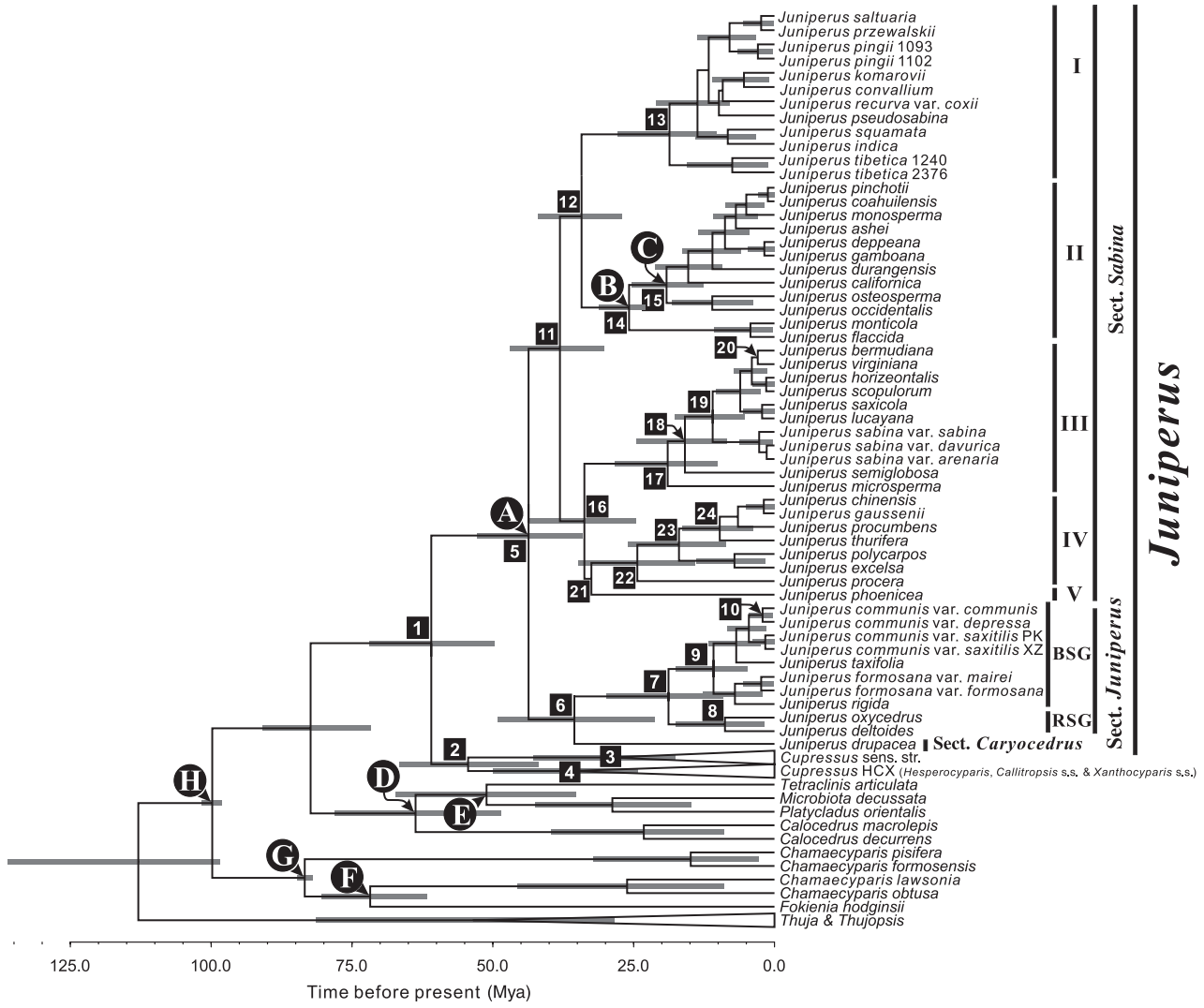

Fig. 3 The divergence time-scale of *Juniperus* derived from BEAST. Dark grey bars represent 95% credible intervals for each node, while white triangular bars (with black outline) represent compressed clades. Letters in black circles represent fossil calibration points (see Table 1), and numbers in black squares indicate numbers for nodes of interest (see Table 2).

Results

Phylogenetic analyses

All sequences determined in this study were submitted to GenBank (HM023885–HM024705 and HM001193–HM001196). Within Cupressaceae sensu stricto, the lengths of the *rbc*L and *psb*B_1-B_2 regions were 1280 and 1349 bp, respectively, for all taxa, whereas the *IGS mat*K, *pet*B-D, *trn*S-G, *trn*D-T, *trn*V intron, *trn*L-F and *rps*4 regions all contained indels and were thus of variable lengths (Table S4). The sequences were aligned and concatenated together, generating a matrix of 10 299 characters, of which 8981 were constant and 1318 were variable; of these variable characters, 416 were parsimony-uninformative and 902 were parsimony-informative (Table S4). For the MP analysis, indels were also coded as additional characters weighted the same as a substitution, so the matrix for this analysis contained 10 677 characters, of which 8983 were constant and 1694 were variable; of these variable characters, 521 were parsimony-uninformative and 1173 were parsimony-informative. Where two accessions were identical for all data, only one was included in the analysis. Because some accessions only differed from others in indel characters, this meant that 116 accessions were included in the MP analysis, whereas for all other analyses the data set was reduced to 92 accessions.

The topologies from the MP and Bayesian analyses were congruent, except that a few small groupings received MP bootstrap support values between 56% and 90%, but lacked

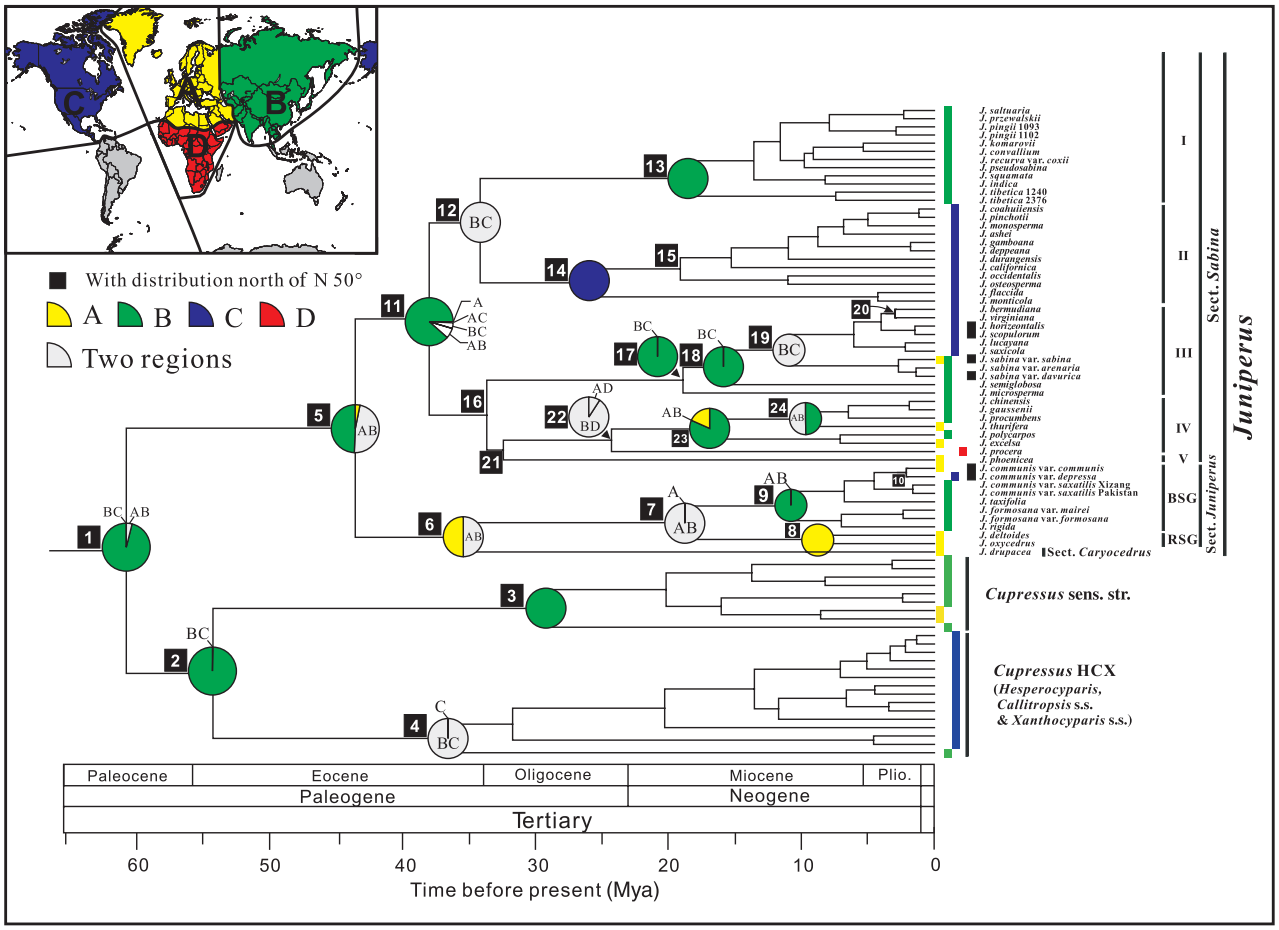

Fig. 4 The results of Bayes-DIVA ancestral area reconstruction analysis. Age ranges for numbered nodes, based on BEAST, MULTIDIVTIME and PLRS, are given in Table 2.

posterior support (Fig. 2). In these analyses, both *Juniperus* and *Cupressus* sens. lat. were monophyletic, and sister to one another, although monophyly of *Cupressus* sens. lat. was only weakly supported (Fig. 2). *Cupressus* sens. lat. comprised two monophyletic clades, of which one comprised *Cupressus* sens. str., as recognized by Little (2006), and the other comprised the other three genera: *Hesperocyparis*, *Callitropsis* sens. str. and *Xanthocyparis* (sens. str.).

Within *Juniperus*, all three sections were strongly supported as monophyletic, although relationships between sections were barely resolved (Fig. 2). Sect. *Juniperus* comprised two well-supported subclades, corresponding to the 'blue seed cone' (BSG) and 'red seed cone' (RSG) groups proposed by Adams (2008a). Sect. *Sabina* comprised five monophyletic clades, among which four (I, II, III and V) had maximum posterior and bootstrap support whereas clade IV had 1.0 posterior and 85% bootstrap support. Clade I contained *Juniperus pseudosabina* from Xinjiang (China) plus all Himalayan/QTP alpine species except *Juniperus microsperma* and *Juniperus gaussenii*. Clade II comprised the serrate-leaved junipers of North America. Clade III comprised the smooth-leaved American species plus the Eurasian *J. sabina*, the middle Asian *Juniperus semiglobosa* and the QTP endemic *J. microsperma*. Clade IV comprised the *Juniperus chinensis* complex from East Asia, *Juniperus thurifera* from Europe, *J. excelsa* from the eastern Mediterranean, *Juniperus polycarpos* (from west Himalaya to Caucasus) and *Juniperus procera* (east Africa and south Arabia). Clade V contained only the Mediterranean *J. phoenicea*, apparently the only Old World *Juniperus* species with truly serrate leaves (Adams, 2008a). Among these clades, I and II were sister to each other with moderate support (MP = 83%; Bayesian posterior possibility = 0.97), but otherwise relationships among them were unresolved (Fig. 2).

The nrITS data set for 24 species comprised 1140 characters (including 39 gap coding characters), of which 937 were constant and 203 were variable; among these variable characters, 119 were parsimony-uninformative and 84 were parsimony-informative. MP analyses suggested that all three sections comprised a monophyletic group, with sects *Juniperus* and *Sabina* sister to one another (Fig. 5a). Within

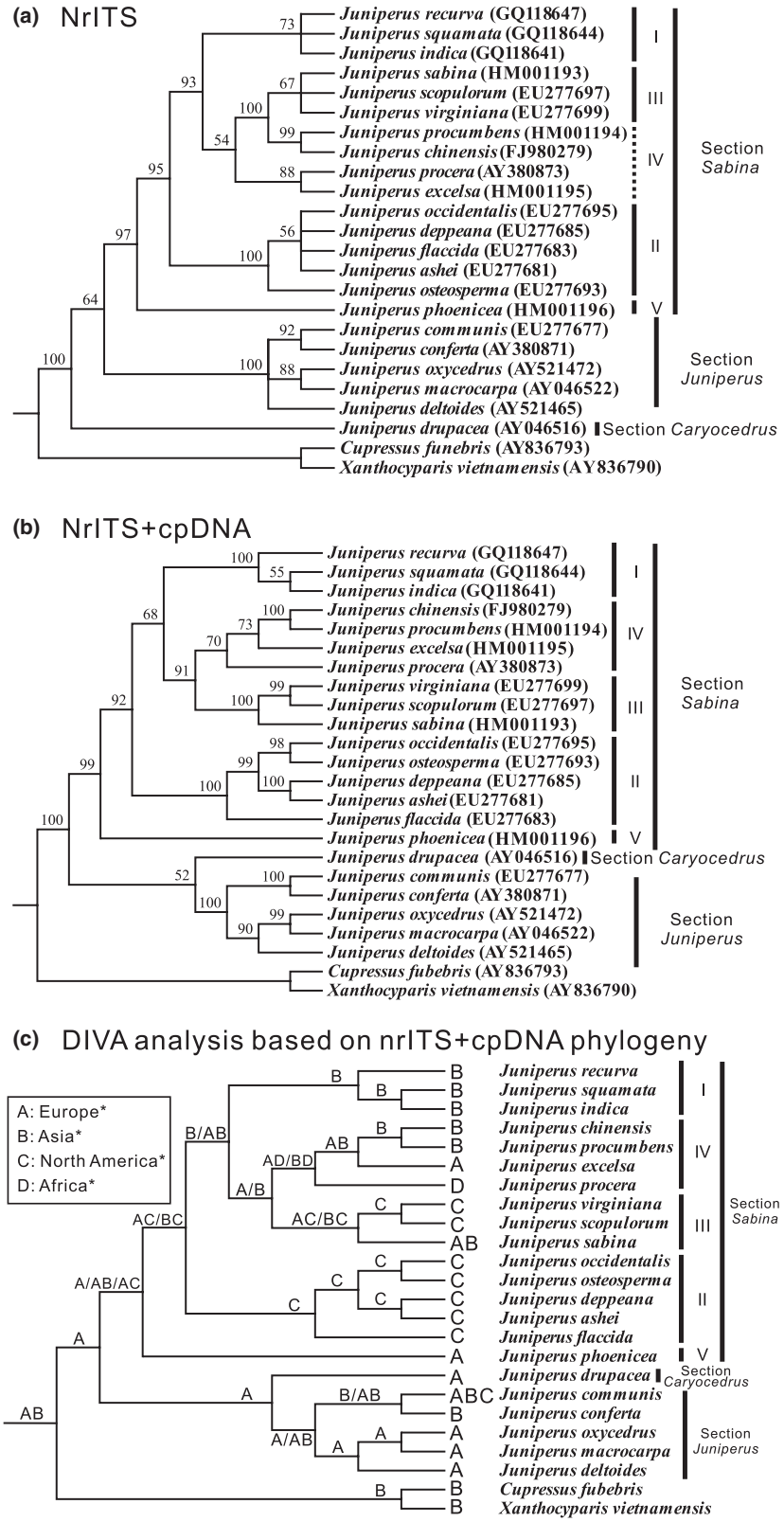

Fig. 5 Molecular phylogenetic relationships among main lineages within *Juniperus* based on nuclear internal transcribed spacer (nrITS) data (a) and nrITS plus cpDNA data (b), and results from the DIVA analysis based on nrITS plus cpDNA phylogeny (c). The GenBank accession number for the nrITS sequence of each species is given in brackets and clade divisions follow cpDNA phylogenies. The vertical dotted line in (a) indicates that *Sabina* clade IV is not supported as monophyletic by nrITS data. *Boundaries among areas are shown in the inset of Fig. 4.

sect. *Sabina*, four cpDNA clades (I, II, III and V) were supported as monophyletic, but clade IV was paraphyletic with respect to clade III; clade V was sister to all others.

A combined analysis of cpDNA and nrITS data produced a tree similar to that for cpDNA alone, but with a better resolution of inter-clade relationships within sect. *Sabina* (Fig. 5b). However, the 'partition homogeneity' test indicated significant contradiction and incongruence between the nrITS and cpDNA data sets ($P = 0.01$).

Timing of divergence events within *Juniperus* and its relatives

In the BEAST analysis, the two combined MCMC runs yielded sufficient effective sample sizes (> 300) for all relevant parameters (e.g. branch lengths, topology and clade posteriors), indicating adequate sampling of the posterior distribution. Levels of rate heterogeneity were high (coefficients of rate variation 0.89). PLRS calculations were carried out under a group of smoothing values between 320 and 3200 which were determined for the 100 bootstrap replicates by cross-validation. Meanwhile, two independent runs of MULTIDIVTIME resulted in practically identical estimation of ages.

Among the three methods used to calculate node ages, PLRS tended to give the youngest ages and MULTIDIVTIME the oldest ages, with BEAST intermediate; however, in all but one case, confidence ranges for the three methods overlapped (Table 2; Fig. S1). Confidence ranges were broadest for MULTIDIVTIME and narrowest for PLRS.

For ease of discussion, summary age ranges for each node were calculated in the form (*W*–) *X*–*Y* (–*Z*), where *W* is the oldest possible age according to any method, and *X* is the oldest possible age according to either of the other two methods; likewise, *Z* is the youngest possible age according to any method, and *Y* is the youngest possible age according to either of the other two methods. This means that the range *X*–*Y* describes the range of node ages that fall within the confidence ranges calculated by at least two of the methods used, and may hence be regarded as the probable age range of that node; the age ranges *W*–*X* and *Y*–*Z* are within the range of one method but outside the age range of the other two, and so are the least likely portions of the age ranges for each node. In general, the probable (*X*–*Y*) age range tended to be similar to the age range indicated by BEAST.

Based on these methods, *Juniperus* diverged from *Cupressus* sens. lat. (75.9–) 71.9–49.7 (–49.7) Mya, that is, during the Paleocene or adjacent periods (node 1: Table 2; Figs 3, 4, S1). The first divergence event within *Juniperus*, which appears to have been the divergence of sect. *Sabina* from the other two sections, occurred about 15 Myr later, in the Eocene or possibly the earliest Oligocene period ((58.7–) 52.9–34.1 (–33.3) Mya; node 5: Table 2; Figs 3, 4, S1). Diversification of sect. *Sabina* into five clades (I–V) occurred from the middle Eocene to the middle Oligocene, apparently in quick succession (Fig. 7), with the first and last events (nodes 11 and 21) occurring (54.8–) 47–30.3 (–27.6) Mya and (51.3–) 37.3–27.4 (–26.2) Mya, respectively (Table 2; Figs 3, 4, S1).

Of three Eurasia–North America disjunctions within *Juniperus*, the first was between Clades I and II of sect. *Sabina*, and occurred during the period mentioned above, that is, (53.1–) 41.9–29.9 (–27.1) Mya. The second, within clade III of sect. *Sabina*, probably arose during the Miocene ((30.6–) 17.6–5.5 (–5.2) Mya; node 19; Table 2; Figs 3, 4, S1). The third and final America–Eurasia disjunction within *Juniperus* involved the American var. *depressa* and the European var. *communis* within *J. communis*, and was much more recent ((14.1–) 4.6–0.3 (–0.1) Mya; node 10; Table 2; Figs 3, 4, S1).

Diversification rates

The average diversification rate within *Juniperus*, calculated from 43.66 Mya (when the first divergence within *Juniperus* took place according to mean node age from the BEAST analysis) to present, was 0.078 speciation events per lineage per million years. However, a Cramer–von Mises test ($W2 = 4.98$, $P < 0.01$) strongly rejected the hypothesis that the diversification rate of *Juniperus* was constant. A stepwise plot of diversification rate through time indicated that the diversification rate of *Juniperus* was above the average during the Eocene, the late Miocene and Pliocene but below the average during the Oligocene, early to middle Miocene and Quaternary periods (grey dotted line; Fig. 6b). These patterns are seen more clearly if the diversification history is artificially divided into four phases (Fig. 6b). If so, the first and fourth phases, 43.66–32 and 11.5-0 Mya, respectively, had diversification rates 37.91% and 39.45%, respectively, higher than the overall average, whereas the intervening periods, 32–20 and 20–11.5 Mya, respectively, had rates 61.60% and 2.70%, respectively, lower than the overall average.

Biogeographic reconstruction

From the Bayes-DIVA analysis (cpDNA), it could be inferred that *Juniperus* and its sister group *Cupressus* sens. lat. share a common ancestor whose ancestral distribution area is probably (*c.* 96%) Asia (Fig. 4). For *Juniperus*, the area of origin could be Europe, Asia, or a combination of these two (Fig. 4). The common ancestor of sect. *Juniperus* was inferred to be in Europe or Asia, whereas that of sect. *Sabina* was probably in Asia (Fig. 4). Overall, the analysis indicated that *Juniperus* diversified within Eurasia, and dispersed to North America via three distinct lineages: *Sabina* clade II, part of *Sabina* clade III, and *Juniperus communis* var. *depressa*.

Table 2 Divergence times for nodes within *Juniperus* and close relatives, based on three rate-smoothing methods that employ relaxed molecular clocks

Node no.	Description of node	Fossil calibration	BEAST (Mya)	MULTIDIVTIME (Mya)	Penalized likelihood rate smoothing (PLRS) (Mya)	Summary date range (Mya)
1	Split between *Cupressus* sens. lat. and *Juniperus*	–	60.93 (49.66–71.90)	65.44 (54.14–75.89)	53.00 (49.68–56.31)	(75.9–) 71.9–49.7 (–49.7)
2	Split between *Cupressus* sens. str. and HCX clade[1]	–	54.39 (41.90–66.63)	63.82 (52.51–74.40)	49.95 (46.17–53.73)	(74.4–) 66.6–46.2 (–41.9)
3	Crown of *Cupressus* sens. str. clade	–	29.30 (17.61–42.78)	39.14 (23.36–54.31)	18.48 (14.71–22.26)	(54.3–) 42.8–17.6 (–14.7)
4	Crown of *Cupressus* HCX clade[1]	–	36.70 (24.20–49.92)	48.28 (34.87–61.25)	31.24 (24.83–37.65)	(61.3–) 49.9–24.8 (–24.2)
5	Crown of genus *Juniperus*	≥ 33.9	43.66 (34.09–52.87)	46.02 (35.48–58.65)	36.02 (33.27–38.77)	(58.7–) 52.9–34.1 (–33.3)
6	Split: sects *Juniperus–Caryocedrus*	–	35.52 (21.22–49.08)	42.46 (31.51–55.23)	33.16 (29.88–36.44)	(55.2–) 49.1–29.9 (–21.2)
7	Crown of sect. *Juniperus*	–	18.82 (9.16–29.89)	29.29 (14.99–45.59)	16.93 (11.14–22.72)	(45.6–) 29.9–11.1 (–9.2)
8	Crown of RSG in sect. *Juniperus*	–	8.79 (1.74–17.46)	19.93 (3.72–37.84)	11.25 (5.69–16.81)	(37.8–) 17.5–3.7 (–1.7)
9	Crown of BSG in sect. *Juniperus*	–	10.83 (4.74–17.47)	19.58 (7.13–36.23)	8.23 (3.93–12.54)	(36.2–) 17.5–4.7 (–3.9)
10	Split: European–NA within *J. communis*	–	2.11 (0.27–4.56)	4.08 (0.12–14.11)	1.83 (0.33–3.33)	(14.1–) 4.6–0.3 (–0.1)
11	Crown of sect. *Sabina*	–	38.09 (30.30–46.99)	42.23 (31.77–54.78)	33.91 (27.64–40.18)	(54.8–) 47–30.3 (–27.6)
12	Split: clade I from II (sect. *Sabina*)	–	34.25 (27.09–41.93)	40.43 (29.93–53.09)	32.93 (30.08–35.79)	(53.1–) 41.9–29.9 (–27.1)
13	Crown of clade I (sect. *Sabina*)	–	19.30 (11.00–28.63)	29.98 (18.28–43.49)	19.85 (14.49–25.20)	(43.5–) 28.6–14.5 (–11)
14	Crown of clade II (sect. *Sabina*)	≥ 23.0	25.82 (23.00–31.20)	29.43 (23.25–41.72)	23.01 (22.88–23.15)	(41.7–) 31.2–23 (–22.9)
15	MRCA of *J. osteosperma* and *J. californica*	≥ 16.0	19.16 (16.00–25.44)	24.39 (15.88–36.64)	16.83 (15.03–18.64)	(36.6–) 25.4–15.9 (–15)
16	Split: clade III from IV and V (sect. *Sabina*)	–	33.75 (24.50–43.61)	40.10 (29.22–52.82)	31.71 (26.17–37.25)	(52.8–) 43.6–26.2 (–24.5)
17	Crown of clade III (sect. *Sabina*)	–	18.96 (10.09–28.28)	24.90 (13.05–38.92)	13.88 (8.81–18.94)	(38.9–) 28.3–10.1 (–8.8)
18	MRCA of *J. semiglobosa* and *J. virginiana*	–	15.90 (8.51–24.54)	22.19 (10.79–36.13)	12.84 (8.81–16.86)	(36.1–) 24.5–8.8 (–8.5)
19	MRCA of *J. sabina* and *J. virginiana*	–	10.97 (5.23–17.60)	16.98 (6.52–30.61)	9.25 (5.54–12.96)	(30.6–) 17.6–5.5 (–5.2)
20	Split: *J. bermudiana* from *J. virginiana*	–	2.97 (N/A)	4.42 (0.17–13.51)	3.25 (0.86–5.64)	(13.5–) 5.6–0.9 (–0.2)
21	Split: clade IV from V (sect. *Sabina*)	–	32.50 (N/A)	38.55 (27.36–51.32)	31.71 (26.17–37.25)	(51.3–) 37.3–27.4 (–26.2)
22	Crown of clade IV (sect. *Sabina*)	–	24.35 (14.04–34.77)	34.03 (22.00–47.49)	25.43 (21.73–29.12)	(47.5–) 34.8–21.7 (–14)
23	MRCA of *J. excelsa* and *J. chinensis*	–	16.96 (8.62–25.94)	26.61 (14.10–41.07)	18.87 (14.74–23.00)	(41.1–) 25.9–14.1 (–8.6)
24	MRCA of *J. thurifera* and *J. chinensis*	–	9.72 (3.74–16.36)	16.92 (5.90–31.71)	10.52 (7.13–13.92)	(31.7–) 16.4–5.9 (–3.7)

[1]HCX clade comprises *Hesperocyparis*, *Callitropsis* sens. str. and *Xanthocyparis* sens. str. within *Cupressus* sens. lat.
Also shown are fossil calibrations (where applied) and summary date ranges (see text).
BSG, blue seed cone group; RSG, red seed cone group; NA, North American; MRCA, most recent common ancestor.

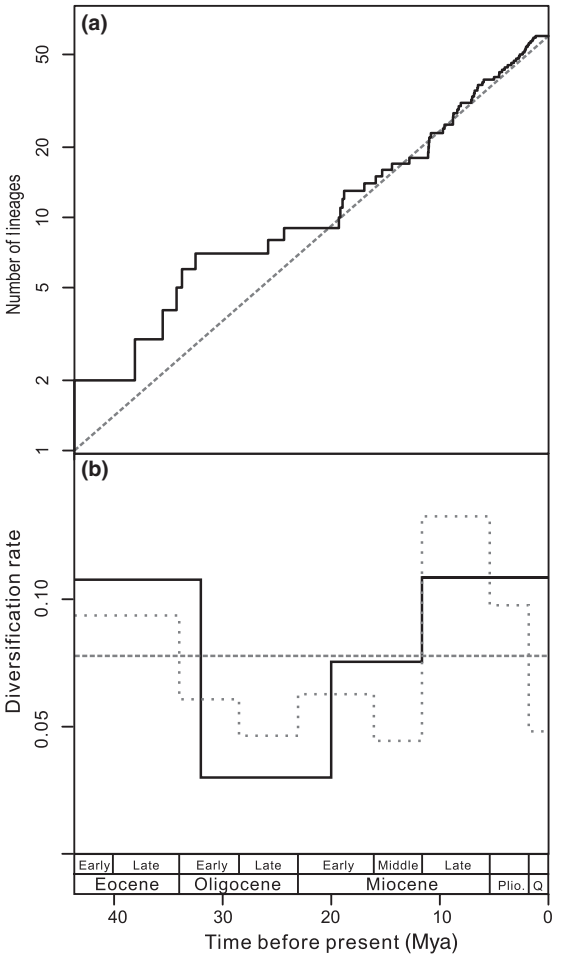

Fig. 6 The lineage through time plot (a), and diversification rate through time plot (b) for *Juniperus*. The grey dashed line in both parts indicates mean net diversification rate; the grey dotted line in (b) represents diversification rate within stratigraphic periods or parts thereof; the black line in (b) represents diversification rates if the history of *Juniperus* is divided into four periods, that is, before 32 Mya, 32–20 Mya, 20–11.5 Mya and 11.5–0 Mya.

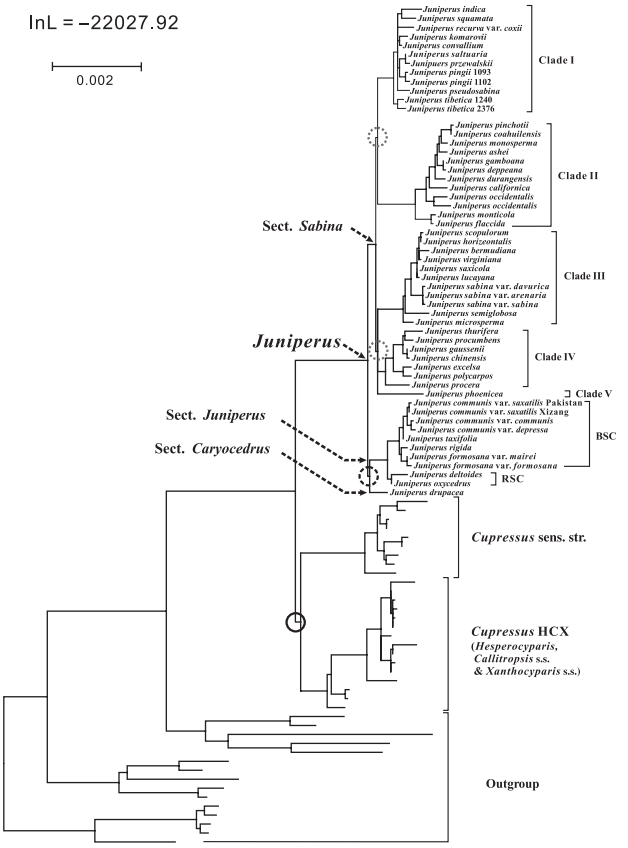

Fig. 7 Maximum likelihood tree generated by GARLI. The solid-line circle, dashed-line circle and grey dotted-line circles indicate short inner branch lengths for inter-genera, inter-section and inter-clade relationships of *Juniperus*, respectively.

DIVA analyses based on a combined (cpDNA + nrITS) phylogeny resulted in similar reconstructions but indicated a probable European origin for both *Juniperus* and its sect. *Juniperus* (Fig. 5c). The earliest divergence event within sect. *Sabina* (the divergence of clade V from all others) also apparently occurred in Europe, although the origin of *Sabina* might have been in Europe or Europe plus another continent (Fig. 5c). The common ancestor of *Juniperus* and *Cupressus* sens. lat. was inferred to be distributed in Eurasia.

Discussion

Diversification history

Our work has generated the most comprehensively sampled and well-resolved phylogeny yet of *Juniperus* and related taxa. Within *Juniperus*, three well-supported monophyletic clades were resolved, corresponding exactly with the three sections recognized by Adams (2004, 2008a), that is, sects *Juniperus*, *Sabina* and *Caryocedrus*. Relationships within sect. *Juniperus* were generally well resolved, and supported a division of the section into two clades corresponding to the BSG and RSG proposed by Adams (2008a). Within the former group, *J. communis* occupies a derived position, indicating that the exceptionally widespread distribution of this species (Fig. 1) is a relatively recent development. Sect. *Sabina* is composed of five strongly supported monophyletic clades; however, in common with previous studies (Little, 2006; Adams, 2008a), relationships between these were mostly unresolved (Figs 2, 5, 7). Although a combined analysis of both cpDNA and nrITS data does partly improve this phylogenetic resolution (Fig. 5b), this result is tentative because the two data sets are not entirely congruent.

Molecular dating based on this cpDNA phylogeny revealed that *Juniperus* diverged from *Cupressus* sens. lat. (75.9–) 71.9–49.7 (–49.7) Mya, and began to diversify (58.7–) 52.9–34.1 (–33.3) Mya, that is, 15-20 Myr later. Hence, during the earliest Tertiary period, the distinctive features of the genus arose, but either no diversification

occurred or all but one of the diverging lineages died out. The acquisition of 'berry-like' seed cones, increasing dispersibility, could have promoted allopatric speciation, as well as permitting rapid range shifts in response to climate change (Farjon, 2005). Indeed, over the next 15 Myr (i.e. until (51.3–) 37.3–27.4 (–26.2) Mya; node 21; Figs 3, 4, S1), *Juniperus* diverged into seven lineages, that is, sects *Juniperus*, *Caryocedrus*, and the five clades of sect. *Sabina*.

Juniperus appears to have gone through phases of slower and faster diversification during the middle Tertiary; using the BEAST node ages without confidence ranges for simplicity, diversification rates within this genus during the periods 43.66–32 and 20–11.5 Mya were much higher than during the intervening period of 32–20 Mya (Fig. 6). This phase of slow diversification within *Juniperus* corresponds closely with a period of relatively stable, cool global temperatures during the early to middle Oligocene (Zachos *et al.*, 2001; Mosbrugger *et al.*, 2005; Miller *et al.*, 2008), whereas the periods either side of this saw falling global temperatures (Eocene) and a brief increase (end Oligocene). The Eocene cooling resulted in the poleward expansion of conifers (Farjon, 2005). Therefore, diversification in *Juniperus* might have been promoted by changes in global and local climates, leading to new adaptations and/or range expansions, but suppressed by long periods of stable climate, as proposed for other Tertiary floras (Milne & Abbott, 2002). However, molecular dates are never exact, and the possibility that the phase of slow diversification occurred just before, or just after, the stable cool period of the Oligocene must therefore be acknowledged.

The rate of diversification within *Juniperus* reached a high point around the late Miocene, and remained high relative to the Oligocene until at least the late Pliocene (Fig. 6b). About half of the 25 Asian species of this genus occur on the QTP, so one contributing factor in this fast diversification phase has certainly been the uplift of the QTP, which began *c.* 40 Mya with two extensive uplifts *c.* 20 and 8 Mya, generating a range of novel ecological niches (Harrison *et al.*, 1992; Chung *et al.*, 1998; Guo *et al.*, 2002; Spicer *et al.*, 2003). Diversification of *Sabina* clade I, all but one of whose species occur on the QTP, was mostly within the past 15 Myr and hence matches this time-scale, and also that of other plant radiations within the QTP (e.g. Liu *et al.*, 2006; Wang *et al.*, 2009).

On a broader scale, progressive cooling of the earth from the Miocene onwards appears to have generated increasing amounts of dry habitats of the type favoured by *Juniperus* (Zachos *et al.*, 2001; Farjon, 2005), and promoted diversification (Cavender-Bares & Holbrook, 2001; Wright *et al.*, 2001; Willson *et al.*, 2008; Opgenoorth *et al.*, 2010). The most extreme example of this is the evolution of *J. communis*, which grows well within the modern Arctic Circle (Fig. 1).

Geographical origins, dispersal, and vicariance

Ancestral area reconstruction through Bayes-DIVA analysis based on cpDNA phylogeny indicated that both *Cupressus* sens. lat. and *Juniperus* originated in Eurasia, with Asia strongly favoured for *Cupressus* sens. lat. The same analysis indicated that *Juniperus* could have been distributed across Europe, Asia, or both at the start of its history. However, DIVA analysis based the combined (cpDNA + nrITS) phylogeny indicated an origin of this genus in Europe (Fig. 5c). Furthermore, the presence of the endemic section *Caryocedrus*, both groups of sect. *Juniperus* and the basal clade of sect. *Sabina* (clade V) in the Mediterranean (Farjon, 2005; Adams, 2008a) makes this region a plausible area of origin for *Juniperus*. Moreover, these above evidences support our belief that *Juniperus* is certainly of Old World origin, and appears to have colonized the New World via three discrete lineages. These are, in decreasing order of age, clade II of sect. *Sabina*, part of clade III of sect. *Sabina*, and var. *depressa* of *J. communis* (sect. *Juniperus*). Within the Old World, there has been a single migration from Europe to Africa, and movements between Asia and Europe have occurred in *J. sabina* (Asia to Europe, and is currently of Eurasian distribution), sect. *Sabina* clade IV (two migrations, but their nature is uncertain) and sect. *Juniperus* (at least one migration between Europe and Asia), and, in addition to this, *J. communis* is circumboreal. These long-distance movements may have benefited from the evolutionary innovation of berry-like seed cones of *Juniperus*, which can be dispersed by birds over long distances (Holthuijzen & Sharik, 1985; Santos *et al.*, 1999; Farjon, 2005; Adams, 2008a).

The Bermuda-endemic *Juniperus bermudiana* diverged from *Juniperus virginiana* (the mainland sister species distributed over southeastern North American, which is at least 1350 km away) Mya. Bermuda has never been connected to a landmass, and might have emerged during the Oligocene, though its history is complex (Vogt & Jung, 2007). However, the Bermuda sand dune systems on which *J. bermudiana* grows developed < 1 Mya (Bryan & Cady, 1934; Cox, 1959; Herwitz, 1992; Adams *et al.*, 2008b) which fits the youngest dates in the above age range, although of course the species might predate the formation of this habitat. Similarly, the presence of endemic *Juniperus brevifolia* and *Juniperus cedrus* on the oceanic islands of the Azores and the Canaries, which were never connected to the nearest continental landmasses, respectively 1500 and 100 km away, must be a result of long dispersal. If we assume that the divergence of *J. procera* from its relatives coincided with this species reaching Africa, then this event occurred around the Oligocene, that is, (47.5–) 34.8–21.7 (–14) Mya, at a time when there was no land connection between Africa and Europe (Coryndon & Savage, 1973; Raven & Axelrod, 1974) and their floras were effectively isolated from one another (Potts & Behrensmeyer, 1992).

Long dispersal is also a possible explanation for within-landmass disjunctions, such as that between the Mediterranean *J. thurifera* and a clade of East Asian species (i.e. *J. chinensis* and *J. procumbens*), which arose around the Miocene period, (31.7–) 16.4–5.9 (–3.7) Mya, although this disjunction might also have arisen via a connecting belt of warm-temperate vegetation (Tiffney & Manchester, 2001).

According to our data, the circumboreal distribution of *J. communis* was derived rather recently. Curiously, European material of *J. communis* (var. *communis*) is more closely related to American (var. *depressa*) than to Asian material (var. *saxatilis*; Fig. 2), probably implying a transatlantic dispersal event that occurred (14.1–) 4.6–0.3 (–0.1) Mya, when no transatlantic land connections existed. In common with other circumboreal high-latitude species (e.g. Abbott *et al.*, 2000), *J. communis* may have a complex phylogeographic history that merits closer investigation.

In contrast, the two earlier colonizations of America by *Juniperus* might have been via land connections. One was between clade II of sect. *Sabina* and its Eurasia relatives. Within *Sabina*, clades I and II diverged (53.1–) 41.9–29.9 (–27.1) Mya, and their common ancestor split from clade V (54.8–) 47–30.3 (–27.6) Mya. Throughout these periods, the BLB was certainly available, and probably also the NALB (McKenna, 1983; Tiffney, 2000; Tiffney & Manchester, 2001; Milne & Abbott, 2002). Bayes-DIVA analysis based on cpDNA (see Fig. 4) was consistent with both migration routes, while DIVA analysis based on nrITS plus cpDNA (Fig. 5c) favoured the NALB route. In common with most species of *Juniperus*, members of clade II occur in warm habitats, whereas those species preferring colder conditions, for example *J. communis*, *J. horizontalis* and *Sabina* clade I (Adams, 2004, 2008a; Farjon, 2005), appear to be recently derived, that is, since the Miocene (Figs 2–5). Given that the BLB occupied higher latitudes than the NALB and was consequently colder (Tiffney & Manchester, 2001), the NALB might be a more likely route for clade II. However, another Old World–New World disjunction occurred within sect. *Sabina* clade III (30.6–) 17.6–5.5 (–5.2) Mya, a date range too young for the NALB yet very consistent with vicariance across the BLB, possibly as a result of climate cooling in Beringia before 8 Mya (Wolfe, 1978; Tiffney & Manchester, 2001; Milne & Abbott, 2002). The high latitudes of the BLB were unlikely to represent a serious barrier to clade III (sect. *Sabina*), whose modern members *J. sabina* and *J. horizontalis* still occur north of 55°N and 60°N, respectively.

A Madrean-Tethyan tale for *Juniperus*?

Most species of *Juniperus* are found in warm temperate semi-arid habitats, such as occur in the Mediterranean and North America. Those members of *Juniperus* that are tolerant of the colder conditions found in modern high latitudes and the high-altitude QTP are recently derived (Fig. 4) or occupy derived positions (e.g. clade I; Fig. 5), indicating that warmer habitats are ancestral within *Juniperus*. This is consistent with a hypothesis that some species, and perhaps the whole genus, are remnants of the Madrean-Tethyan vegetation belts, which contained sclerophyllous species adapted to warm temperate semi-arid habitats. These ran along the southern areas of Eurasia and North America during the Eocene and Oligocene, and might have been continuous according to some authors (Axelrod, 1975; Wen & Ickert-Bond, 2009).

Although our ancestral area reconstruction analyses could not conclusively pinpoint Europe or Asia as the exact location of most *Juniperus* lineages during the Eocene and Oligocene, the presence of sect. *Sabina* in central Europe in the late Eocene is proved by the fossil *Juniperus pauli* (Kvaček, 2002). Furthermore, we know that many groups now disjunct between East Asia and North America went extinct in Europe during the late Tertiary (Milne & Abbott, 2002). If this also occurred in *Juniperus*, it might have skewed the Bayes-DIVA analysis of cpDNA data to overestimate the likelihood of Asia for some clades at the expense of Europe. Therefore, as suggested by analyses of combined (nrITS+cpDNA) data sets (Fig. 5), the early divergences of sect. *Sabina* might have occurred in Europe and the direct ancestors of the other clades of this section may have existed in the Tethyan vegetation there, despite leaving no extant descendents today.

Juniperus diverged from *Cupressus* sens. lat. some time before the formation of Madrean-Tethyan vegetation, but the first phase of *Juniperus* diversification coincided with the proposed formation of Madrean-Tethyan vegetation during the Eocene (Axelrod, 1975; Wen & Ickert-Bond, 2009). Similarly, the slow *Juniperus* diversification phase that followed could be linked to a phase when the Madrean-Tethyan vegetation's composition, like the climate it experienced, was stable (Zachos *et al.*, 2001). The first colonization of America by *Juniperus* (sect. *Sabina* clade II) occurred in the middle of the Madrean-Tethyan vegetation's lifespan, and so is entirely compatible with a hypothesis that *Juniperus* was one of many genera from this vegetation that achieved amphi-Atlantic distributions as part of a Madrean-Tethyan vegetation belt. Sect. *Sabina* clade III did not reach North America until the Miocene, and sect. *Juniperus* later still, whereas sect. *Caryocedrus* is now endemic to the Mediterranean. Our results therefore support a diversification of *Juniperus* in the late Eocene Tethyan vegetation, but only one incursion into the American Madrean vegetation.

Conclusions

Juniperus is primarily a genus of mild, semi-arid habitats, and its timing and pattern of diversification fits well with

the idea that it began diversifying as part of the Eocene Tethyan vegetation belt of southern Eurasia. It reached America once during this time, then again during the Miocene and a third time via the now circumboreal *J. communis*. Diversification rates appear to be linked to periods of ongoing global climate change, although specific events such as the uplift of the QTP were also important. Dispersal has certainly, and vicariance very probably, contributed to its current distribution across Europe, Asia, North America and Africa.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Fig. S1 Divergence times for nodes within *Juniperus*.

Notes S1 Fossil calibrations.

Table S1 Sample provenance and collection IDs.

Table S2 Primers and their provenance.

Table S3 PCR programmes employed for each primer pair.

Table S4 Sequence lengths and indel numbers.

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