A Total Evidence Approach to Understanding Phylogenetic Relationships and Ecological Diversity in *Selanginella* subg. *Tetragonostachys*

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A TOTAL EVIDENCE APPROACH TO UNDERSTANDING PHYLOGENETIC RELATIONSHIPS AND ECOLOGICAL DIVERSITY IN *SELAGINELLA* SUBG. *TETRAGONOSTACHYS*[1]

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- *Premise of the Study:* Several members of *Selaginella* are renowned for their ability to survive extreme drought and "resurrect" when conditions improve. Many of these belong to subgenus *Tetragonostachys*, a group of ~45 species primarily found in North and Central America, with substantial diversity in the Sonoran and Chihuahuan Deserts. We evaluated the monophyly and the age of subgenus *Tetragonostachys* and assess how drought tolerance contributed to the evolution of this clade.
- *Methods:* Our study included most *Tetragonostachys* species, using plastid and nuclear sequences, fossil and herbarium records, and climate variables to describe the species diversity, phylogenetic relationships, divergence times, and climatic niche evolution in the subgenus.
- *Key Results:* We found that subgenus *Tetragonostachys* forms a monophyletic group sister to *Selaginella lepidophylla* and may have diverged from other *Selaginella* because of a Gondwanan–Laurasian vicariance event ca. 240 mya. The North American radiation of *Tetragonostachys* appears to be much more recent and to have occurred during the Early Cretaceous–late Paleocene interval. We identified two significant and nested ecological niche shifts during the evolution of *Tetragonostachys* associated with extreme drought tolerance and a more recent shift to cold climates. Our analyses suggest that drought tolerance evolved in the warm deserts of southwest North America and may have been advantageous for colonization of cold and dry boreal climates.
- *Conclusions:* Our investigation provides a foundation for future research addressing the genomics of ecological niche evolution and the potential role of reticulate evolution in *Selaginella* subgenus *Tetragonostachys*.

Key words: Ecological niche shift; lycophyte; Ornstein-Uhlenbeck models; *Selaginella*; Sonoran Desert.

With ~700 species, *Selaginella* P. Beauv. is one of the largest genera of vascular plants (Jermy, 1956, 1990) and one of the earliest-diverging lineages of extant vascular plants, with an origin dating to ca. 400 mya (Banks, 2009). As the largest group of heterosporous, non-seed plants, *Selaginella* yields key insights into plant evolution, and the recent release of the *S. moellendorfii* genome (Banks et al., 2011) opens the door to understanding the development of critical land plant features such as the vascular system and obligate heterospory. Furthermore, *Selaginella* species are outstanding for their small genome sizes and lack of ancient polyploidy (Banks et al., 2011), a prominent feature of all other surveyed vascular plant genomes (e.g., Cui et al., 2006; Soltis et al., 2009; Arrigo and Barker, 2012). Many species of *Selaginella* have smaller nuclear genomes than *Arabidopsis* (157 Mb), with the recently sequenced *S. moellendorfii* genome at 106 Mb (haploid complement; Banks et al., 2011) and flow cytometry estimations (Little et al., 2007) ranging between 84 Mb (*S. apoda*) and 240 Mb (*S. kraussiana*). This feature makes *Selaginella* species particularly amenable to *de novo* genome assembly from data gathered using next-generation sequencing.

Numerous aspects of evolution and ecological diversity remain to be explored in *Selaginella.* Historically, this large genus has been organized into smaller subgenera based on shared morphological character states. The present study addresses the phylogenetic and ecological diversity of subgenus *Tetragonostachys*, a clade of ca. 46–50 species with a center of diversity in the deserts of southwestern North America (Tryon, 1955; Valdespino, 1993). This subgenus is characterized by spirally arranged, isophyllous to anisophyllous microphylls, tetrastichous sporophylls, and the presence of dorsal rhizophores (Korall and Kenrick, 2004). The subgenus also includes the only *Selaginella* representatives known to have a vascular system partly composed of vessels (Duerden, 1934; Harvey-Gibson, 1894). Species of subgenus *Tetragonostachys* are of particular interest in studies of drought tolerance because many show clear adaptations to xeric habitats. For example, several species possess microphylls oriented to reduce surface exposure to direct sunlight. Other so-called "resurrection" species show extreme drought-resistance adaptations, with stems preventing overheating and dehydration by curling inward when deprived

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of water and unfurling when water is available after extended periods of dormancy.

Although two studies have examined the phylogeny of *Selaginella* (Korall and Kenrick, 2002, 2004), none has extensively sampled the diversity of subgenus *Tetragonostachys*. As a result, little is known about the phylogeny and evolution of these numerous drought-tolerant species, in particular whether they form a natural, monophyletic group or are classified together because of convergent evolution to xeric habitats. Here, we aim to fill this gap by analyzing plastid and nuclear DNA sequences from nearly every extant species of subgenus *Tetragonostachys* to assess monophyly and clade ages. On the basis of accessibile sequences from published analyses (Korall and Kenrick, 2002, 2004) and the availability of a large, previously unpublished collection of sequences in GenBank for subgenus *Tetragonostachys*, we reconstructed relationships using *rbcL* and ITS sequences. Coupled with a broad survey of georeferenced herbarium collections and meta-analyses of publicly available ecological data, these data allowed us to test whether drought tolerance is more prevalent among members of this subgenus than among other clades of *Selaginella*. Overall, our analyses provide a representative phylogeny and divergence time estimates for the subgenus and explore the evolution of ecological niches within the subgenus that will be valuable for future studies of drought tolerance.

MATERIAL AND METHODS

Plant material, sequences, and herbaria—Specimens of 40 of the 46–50 taxa included in the subgenus *Tetragonostachys* (Tryon, 1955; Valdespino, 1993) were obtained, either as living collections (Pima County, Arizona, USA) or as herbarium material. The sampling was representative of both the morphological and geographic diversity of the subgenus. Total genomic DNA was isolated using Qiagen DNeasy plant extraction kits following the manufacturer's protocols. The amplification of *rbcL* and ITS was conducted in 50-μL volumes, using Promega *Taq* polymerase and reagents according to the manufacturer's protocol (Promega, Madison, Wisconsin, USA), with the addition of 1 μM of BSA. The same PCR program was used for *rbcL* and ITS and included an initial denaturation of 2 min at 95°C, followed by 30 cycles of 1 min at 95°C, 1 min at 42°C, 3 min at 72°C, and a final elongation of 8 min at 72°C. The *rbcL* region was amplified with primers described in Korall and Kenrick (2002), whereas ITS required the use of specific primers designed during a preliminary study (5′-TCCGTAGGTGAACCTGCGGAAGGA-3′ and 5′-TCCTCCGCTTATTGATATGCTTAAACT-3′). DNA sequencing was performed on an ABI 310 capillary sequencer (Applied Biosystems, Foster City, California, USA), following the manufacturer's protocol. The sequences were deposited in GenBank under the accession numbers AF419048 to AF419090 for *rbcL* and AF418999 to AF419047 for ITS.

We collected herbaria records from the Global Biodiversity Information Facility, Calflora, Consortium of California Herbaria and the University of Arizona Herbarium databases to build a comprehensive set of georeferenced occurrences for *Selaginella* species included in the current study. Specimens with imprecise or missing geographic locations were either discarded or assigned new coordinates using gazetteers and Google Earth. The resulting database was used to develop hypotheses about the evolution of ecological niches in *Selaginella* as a whole (see below), and assess the worldwide species diversity and ecological niche evolution of *Tetragonostachys* in particular. Species diversities were computed from a five decimal-degree grid and visualized with Google Earth, using functions implemented in the R2G2 package (Arrigo et al., 2012).

Phylogenetic analyses—First, we investigated the monophyly of subgenus *Tetragonostachys* and its relationships to other species of *Selaginella*. The *rbcL* data set included all available *Selaginella* sequences retrieved from GenBank (Appendix S1, see Supplemental Data with the online version of this article) and five outgroup taxa (i.e., *Huperzia selago*, *Lycopodium annotinum*, *L. digitatum*, *Isoëtes lacustris*, and *I. melanopoda*) representing the other major clades of lycophytes. Second, we focused on *Tetragonostachys* by investigating the *rbcL* and ITS data sets separately. Sequence alignments were performed using SATe (Liu et al., 2009) and manually corrected following a similarity-based strategy (Simmons, 2004) and using BioEdit (Hall, 1999). The ITS alignment required further editing because it had a large proportion of gaps. We used BMGE (Criscuolo and Gribaldo, 2010), an algorithm that computes local alignment entropy to objectively remove difficult regions of the alignment. The analysis relied on default parameters and a PAM5 similarity matrix to apply a stringent filtering (i.e., 65% of the ITS alignment was discarded). The *rbcL* and ITS alignments were deposited in Dryad (http://dx.doi.org/10.5061/dryad.s44s8). MrModelTest version 2.3 (Nylander, 2004) was used to select the models of molecular evolution. On the basis of Akaike's information criterion, all data sets required the GTR+G+I model. However, because of convergence difficulties encountered when analyzing the *rbcL* phylogeny, we used a simplified model (GTR+G).

Phylogenetic analyses were conducted with MrBayes version 3.1.2 (Ronquist and Huelsenbeck, 2003) with default parameters (two runs with four chains each). Heuristic searches were 20 and 10 million generations long for the whole genus and the ingroup phylogenies, respectively. Trees were sampled each 1000 generations, and the convergence of runs was checked using Tracer version 1.4 (Rambaut and Drummond, 2007). The Potential Scale Reduction Factor (PSRF) criterion was computed as implemented in MrBayes (i.e., PSRF values were all equal to one to three decimal places), and an allcompat consensus tree was generated after discarding 30% of the initial trees produced by each run. The software PRAP version 2.0 (Müller, 2004) was then used to compute decay indexes (Bremer, 1988) for the obtained phylogenies, using parsimony ratchet (Nixon, 1999). PRAP was set as in Buerki et al. (2009), and computations were performed with PAUP* version 4.0b10 (Swofford, 2002). Because the phylogenies obtained using MrBayes and parsimony ratchet were highly congruent, only results of the former approach were displayed. Decay indexes were indicated only for the nodes supported by parsimony ratchet. Finally, the topological congruence between *rbcL* and ITS ingroup phylogenies was estimated by computing quartet distances, using DARwin version 5.0 (Perrier and Jacquemoud-Collet, 2006). All phylogenetic trees were edited with FigTree version 1.3.1 (Rambaut and Drummond, 2009).

Dating—Four fossils were used as reference points in the dating analyses. The split between *Selaginella* and its sister group *Isoëtes* was fixed at 370 mya on the basis of the isoëtalean *Lepidosigillaria* (Bateman et al., 1992; Kenrick and Crane, 1997) found in the Upper Devonian. Three additional fossils could then be placed on different nodes within the *Selaginella* phylogeny based on synapomorphies for particular crown groups (Appendix S2), thus giving the stem node of the extant group a minimum age equivalent to that of the fossil.

The minimum age assigned to the rhizophoric clade, and hence to crown group Selaginellaceae, was set to 310 mya on the basis of the Lower Carboniferous *Selaginella suissei* (Korall and Kenrick, 2004). The Late Triassic *S. anasazia* (Ash, 1972) provides a minimum age of 210 mya for the stem group of the clade consisting of *S. remotifolia*, *S. kraussiana*, *S. sericea*, *S. articulata*, *S. lingulata*, *S. diffusa*, *S. sulcata*, *S. suavis*, and *S. fragilis*. Finally, megaspore fossils of the genus *Erlansonisporites* have been reported from many localities ranging from Triassic to Cretaceous in age (Takahashi et al., 2001). They have a combination of megaspore characters found only in a small clade of three species in the extant phylogeny, including *S. lyallii*, *S. polymorpha*, and *S. moratii* (Korall and Taylor, 2006). *Erlansonisporites scanicus* thus gives a minimum age constraint for the stem group of these three species of 85 mya.

Three approaches were used for the dating analyses: nonparametric smoothing as implemented in PATHd8 version 1.9.8 (Britton et al., 2007), penalized likelihood (PL) implemented in r8s version 1.8 (Sanderson, 2002, 2003) and a Bayesian framework implemented in BEAST software version 1.7.3 (Drummond et al., 2012). PATHd8 is a nonparametric smoothing method that minimizes the differences in rates between sister groups, as opposed to other autocorrelation-based smoothing methods. PL is a semiparametric smoothing method that combines a model-based likelihood with a roughness penalty, regulated by a smoothing parameter. To obtain an optimal smoothing value, we used the fossil-based cross validation implemented in r8s (Near and Sanderson, 2004). The clock test for individual nodes in PATHd8 rejected the clock hypothesis for almost half of the nodes (95% confidence). For both PATHd8 and PL, confidence intervals were based on 1000 randomly chosen phylograms from the MrBayes output, after burn-in of 25% and filtering of the trees to ensure congruence with the fossil constraints. Except for the enforced monophyly of fossil-constrained groups, this approach takes into account uncertainty in both topology and branch lengths. The dating analyses of these 1000 trees were summarized using TreeAnnotator (part of the BEAST package; Drummond et al., 2012), using the dated majority-rule consensus tree as target tree.

The BEAST analysis is detailed in Appendix S2. In short, the method performs the simultaneous estimation of tree topology and divergence times while accounting for uncertainty in fossil placements. Only the results obtained with PL are displayed here, with divergence-time estimates obtained from PATHd8 and BEAST provided in Appendix S3. Nexus files describing these phylogenetic trees are deposited in Dryad (http://dx.doi.org/10.5061/dryad.s44s8).

Ecological niche evolution—Shifts in ecological niche were modeled in two separate analyses (see above), one involving the complete set of *Selaginella* species and the other restricted to the members of subgenus *Tetragonostachys*. Both analyses followed the same procedures but relied on the *rbcL* phylogeny (dated with PL) or on undated ITS trees (see below), respectively. The ecological niches of extant species were described by 19 climatic variables (BioClim; Hijmans et al., 2005a) reflecting temperature and precipitation regimes (Appendix S4) retrieved for all available herbarium records using the DIVA-GIS freeware (Hijmans et al., 2005b). Because these climatic variables were highly correlated with each other, we performed principal component analyses (PCA) to summarize them as highly explanatory eigenaxes. The PCA were performed after standardizing the 19 BioClim variables (i.e., subtraction of mean followed by variance division, as implemented in R CRAN; R Development Core Team, 2012) and focused either on all *Selaginella* species or only on subgenus *Tetragonostachys*. The calculated eigenaxes differed slightly according to the taxonomic range considered in each ecological subset (see results). These geographic occurrences and corresponding BioClim data are deposited in Dryad (http://dx.doi.org/10.5061/dryad.s44s8).

Using the picante package (Kembel et al., 2010), we mapped the eigenaxes to their corresponding phylogenies (i.e., by computing the average eigenaxis value of each species) to further test models of ecological niche evolution. Ornstein-Uhlenbeck (OU) models (Hipp, 2007) were applied to assess the significance of ecological niche shifts within the *Selaginella* and *Tetragonostachys* phylogenies. Models of niche shifts varied according to the data set. For the complete *Selaginella* data set, we tested whether ecological niche shifts occurred at the origin of five taxonomically, geographically, and phylogenetically well-supported clades (node 1—origin of *Selaginella* clade B; node 2—*Selaginella* clade A; node 3—clade A species from the Pacific; node 4—clade A from South America; and node 5—*Tetragonostachys* + *S. lepidophylla*). The analysis considered unique niche shifts (i.e., not accounting for niche reversions because they did not affect results; data not shown), along with two null models (i.e., OU model with a single-optimum and pure Brownian motion). The best model was selected on the basis of weighted Bayesian information criterion (BICwi). The analyses were performed using methods implemented in the Maticce R CRAN package (Hipp and Escudero, 2010) and following the manual's recommendations. Phylogenetic uncertainty was incorporated by considering all dated *rbcL* trees that included the five clades discussed above (a total of 541 trees out of 1000 generated with PL dating). The analysis of ecological shifts within subgenus *Tetragonostachys* essentially followed the same procedure, differing only in the used phylogeny and the definition of nodes where ecological shifts were expected. Here, we aimed at statistically testing whether any secondary ecological shifts had occurred within subgenus *Tetragonostachys*. This latter analysis was based on the ITS data set because only this region showed enough phylogenetic signal among *Tetragonostachys* species. Because of poor resolution for most deep relationships, we focused on a highly supported subclade (hereafter clade X; see Results) that was representative of the ecological diversity of subgenus *Tetragonostachys*. We used the same strategy as above, except that the ITS phylogenies were not dated, owing to a lack of accurate fossil calibrations within *Tetragonostachys*. Although ultrametricity is not strictly required by Maticce (Hipp and Escudero, 2010) and is not expected to affect significance assessments of ecological niche shifts, this limitation restricts the interpretation of model parameters that are not considered here (i.e., selection and drift rates). On the basis of qualitative inspection of mapped PCA eigenaxes on phylogenies, only one node was tested for niche-shift significance. Statistical significance was assessed by comparing the cumulative BICwi values of models considering a niche shift (OU with two optima) versus two null models (OU model with single-optimum and pure Brownian motion).

RESULTS

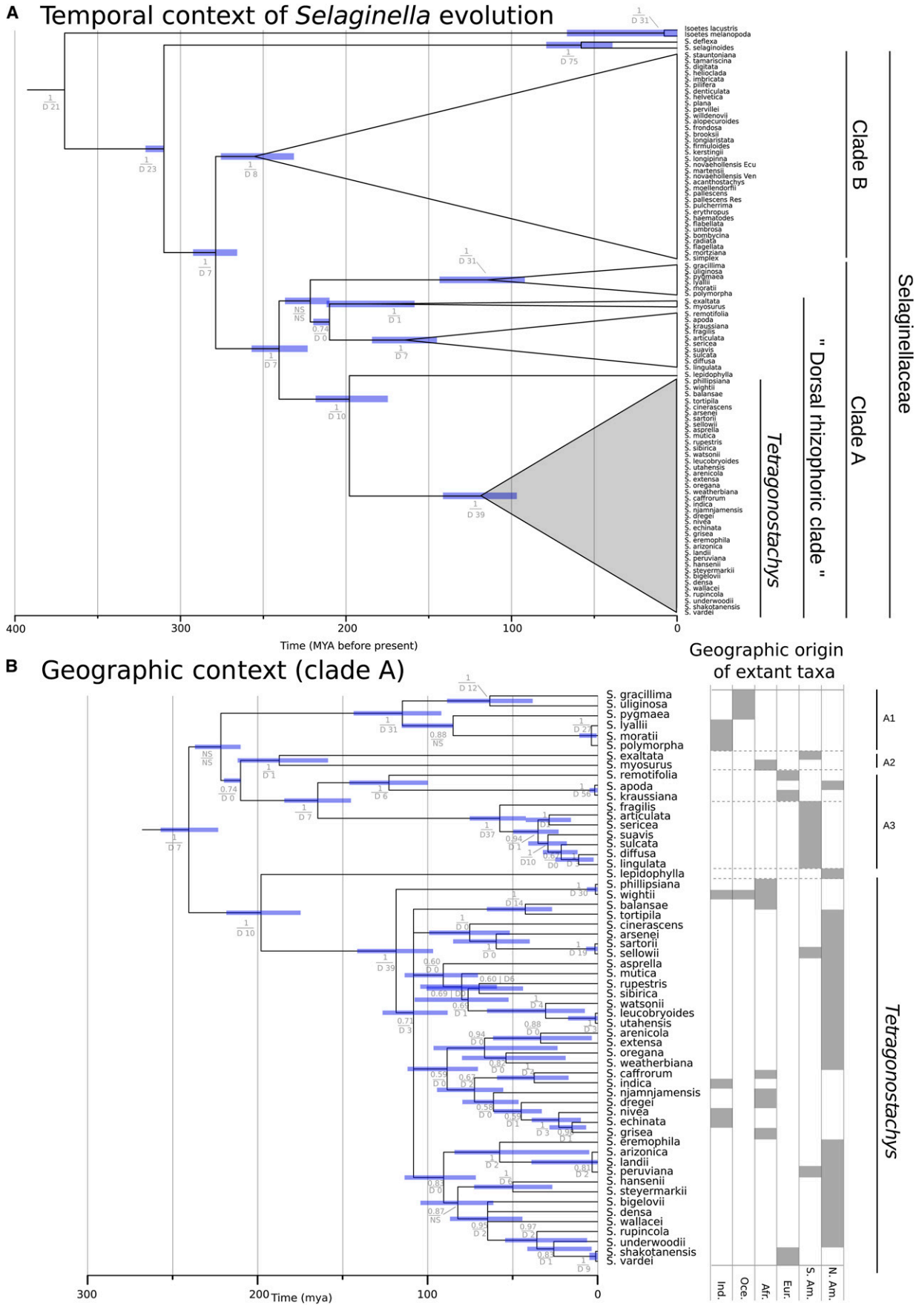
***Phylogeny and divergence time estimates for* Selaginella *and subgenus* Tetragonostachys**—The *rbcL* alignment for the complete *Selaginella* data set was 1274 bp in length, with 593 (46%) parsimony-informative characters (PIC) out of 723 variable sites.

Subgenus *Tetragonostachys* was unambiguously resolved as monophyletic (Fig. 1A), with 39 molecular characters supporting the clade (i.e., decay index [DI]) and a Bayesian posterior probability (PP) of 1. The subgenus was nested in clade A with high confidence (DI = 7 and PP = 1), and *S. lepidophylla* (i.e., the Rose of Jericho) was well supported as sister to subgenus *Tetragonostachys* (DI = 10, PP = 1). The remaining clade A species included three subclades (A1, A2, and A3 in Fig. 1B), all of which had PP = 1 and two of which (A1 and A3) had high decay indexes (31 and 7, respectively). Nevertheless, these three subclades collapsed into a polytomy that made the so-called "dorsal rhizophore clade" (i.e., including all species having rhizophores inserted on the upper surface of shoots) appear paraphyletic or polyphyletic.

The minimum crown-group age of *Selaginella* was 312 Ma (95% confidence interval [CI]: 310–321 Ma) and 322 Ma (95% CI: 310–352 Ma), according to PL and BEAST, respectively (PATHd8 could not estimate the age of this node as it corresponds to a calibration point). The split between clade A and clade B was estimated at 279 mya (95% CI: 266–292 mya), 248 mya (95% CI: 224–270 mya), and 281 mya (95% CI: 247–313 mya) by r8s, PATHd8, and BEAST, respectively. Focusing on clade A (Fig. 1B) revealed an interesting geographic clustering of species. Indeed, the earliest phylogenetic split in clade A segregated species with mostly Central and North American extant distributions (i.e., *S. lepidophylla* and most of subgenus *Tetragonostachys*) from those occurring in South America, Asia, Madagascar, Africa, and Australia. The only exception outside of *Tetragonostachys* was *S. apoda* (belonging to subclade A3), which is widespread in the southeastern portion of North America. Divergence times for this geographic split were comparable across dating methods, with estimates of 240 mya (95% CI: 224–257 mya), 227 mya (95% CI: 224–270 mya), and 239 mya (95% CI: 210–266 mya) generated using r8s, PATHd8, and BEAST, respectively. In stark contrast, divergence-time estimates for subgenus *Tetragonostachys* are strongly dependent on the method applied. Penalized likelihood placed the crown of subgenus *Tetragonostachys* at 108 mya (95% CI: 77–148 mya), the estimate of BEAST was at 100 mya (95% CI: 68–140 mya) whereas PATHd8 gave a much later date of 59 mya (95% CI: 39–88 mya). The diversification of subgenus *Tetragonostachys* occurred during the Early Cretaceous–late

→

Fig. 1. (A) Chronogram of *Selaginella* based on the *rbcL* gene tree. (B) Focus on species from clade A sensu Korall and Kenrick (2002). The geographic origin of extant taxa is indicated (Ind. = India and/or Madagascar, Oce. = Australia, Afr. = Africa, Eur. = Eurasia, S. Am. = South America, N. Am. = central and North America). Phylogenies are allcompat consensus trees inferred by MrBayes. For each node, Bayes posterior probabilities >0.5 appear above the horizontal line, and decay indexes supported by parsimony ratchet (with "D" as suffix) are shown below the line. Estimated node divergence times are portrayed as bars representing 95% confidence intervals computed using penalized-likelihood from 1000 Bayesian trees randomly sampled from posterior tree distribution. Four calibration points were used for dating. The two most external outgroups (*Huperzia* and *Lycopodium* sp.) were pruned off from the *Selaginella* phylogeny.


A
Temporal context of Selaginella evolution
Isoetes lacustris
Isoetes melanopoda
S. deflexa
S. selaginoides
S. stauntoniana
S. tamariscina
S. digitata
S. heliocladata
S. imbricata
S. pilifera
S. denticulata
S. helvetica
S. plana
S. pervillei
S. willdenovii
S. alopecuroides
S. frondosa
S. brooksii
S. longiaristata
S. firmuloides
S. kerstingii
S. longipinna
S. novaehollensis Ecu
S. martensii
S. novaehollensis Ven
S. acanthostachys
S. moellendorffii
S. pallescens
S. pallescens Res
S. pulcherrima
S. erythropus
S. haematodes
S. flabellata
S. umbrosa
S. bombycina
S. radiata
S. flagellata
S. mortziana
S. simplex
S. gracillima
S. uliginosa
S. pygmaea
S. lyallii
S. moratii
S. polymorpha
S. exaltata
S. myosurus
S. remotifolia
S. apoda
S. kraussiana
S. fragilis
S. articulata
S. sericea
S. suavis
S. sulcata
S. diffusa
S. lingulata
S. lepidophylla
S. phillipsiana
S. wightii
S. balansae
S. tortipila
S. cinerascens
S. arsenei
S. sartorii
S. sellowii
S. asprella
S. mutica
S. rupestris
S. sibirica
S. watsonii
S. leucobryoides
S. utahensis
S. arenicola
S. extensa
S. oregana
S. weatherbiana
S. caffrorum
S. indica
S. njamnjamensis
S. dregei
S. nivea
S. echinata
S. grisea
S. eremophila
S. arizonica
S. landii
S. peruviana
S. hansenii
S. steyermarkii
S. bigelovii
S. densa
S. wallacei
S. rupincola
S. underwoodii
S. shakotanensis
S. vardei
Clade B
Clade A
" Dorsal rhizophoric clade "
Tetragonostachys
Selaginellaceae
400
300
200
100
0
Time (MYA before present)
B
Geographic context (clade A)
Geographic origin of extant taxa
Time (mya)
Ind.
Oce.
Afr.
Eur.
S. Am.
N. Am.
A1
A2
A3
Tetragonostachys

Paleocene interval. However, divergence-time estimates within the subgenus remained uncertain, owing to the limited available phylogenetic resolution.

Ingroup phylogenies—The *rbcL* alignment for subgenus *Tetragonostachys* showed 212 (57%) PIC out of 370 variable sites. The initial ITS alignment was 1139 bp long, but phylogenetic analyses were conducted on a filtered alignment where unaligned regions were removed according to an entropy-based criterion. The final ITS data set was thus 393 bp long, with 132 (69%) PIC out of 192 variable characters. This filtering procedure did not markedly influence phylogenetic reconstructions because the obtained trees were highly similar between the initial and the filtered alignments (data not shown). Both sequenced regions showed limited saturation bias due to mutations, as revealed by saturation plots (Appendix S5).

Both DNA regions showed limited resolution for the deepest phylogenetic nodes, and most of the species stemmed from a basal polytomy (Fig. 2). A clearer evolutionary signal was recovered from the ITS analysis, where a highly supported clade (hereafter clade X, DI = 6 and PP = 1) included species from North America and Asia (i.e., *S. hansenii*, *S. steyermarkii*, *S. wrightii*, *S. arizonica*, *S. eremophila*, *S. bigelovii*, *S. wallacei*, *S. rupincola*, *S. watsonii*, *S. densa*, *S. rupestris*, *S. shakotanensis*, *S. sibirica*, and *S. vardei*). This clade was not fully congruent with the topology obtained with *rbcL*, even though the two Asian species (*S. shakotanensis* and *S. vardei*) appeared to be nested among North American species. The ITS and *rbcL* gene trees both showed limited topological congruence (quartet distance = 0.66).

Species diversity and geography of subgenus **Tetragonostachys**—The herbarium data used to estimate ecological niche shifts included 8844 georeferenced *Selaginella* specimens (3715 representing *Tetragonostachys*), with an average of 110 specimens per species (ranging from 3 to 1063 specimens in *S. wildenowii* and *S. selaginoides*, respectively). Members of subgenus *Tetragonostachys* occurred on every continent except Antarctica, with the majority of species (29 of the 40 available) endemic to North and Central America. Exceptions included *S. philipsiana* (Africa), *S. wightii* (Africa, India, and Australia; however, no accurate geographic coordinates were available for the two latter areas), *S. shakotanensis*, and *S. vardei* (Asia), and a weakly supported clade (DI = 2, PP = 0.67) containing the African and Malagasy species *S. caffrorum*, *S. indica*, *S. njamnjamnensis*, *S. dregei*, *S. nivea*, *S. echinata*, and *S. grisea*. Western North America showed the greatest species diversity (Fig. 3 and Appendices S6, S7, and S8, see Supplemental Data with the online version of this article), with evolutionary hotspots along the Pacific coast (Washington, Oregon, and California), in the deserts of the southwestern USA (the Chihuahuan, Sonoran, Mojave, and Great Basin deserts), and in the Sierra Madre and intervening valleys of Mexico.

Ecological range and niche shifts during the evolution of **Tetragonostachys** ***species***—For the complete *Selaginella* data set, the first two PCA eigenaxes explained 41% and 29% of the total variance and respectively outlined seasonal variations of temperature versus absolute temperature and precipitation gradients (Appendix S4). As a result, the ecological-niche-shift model performed at the genus level were focused on the second eigenaxis because it consistently discriminated hot and/or arid from mild and/or humid habitats (Fig. 4).

Drought/heat tolerant species occurred in almost every clade of the phylogeny (Fig. 4); however their prevalence was 3× higher in *Tetragonostachys* (31 of 37 species with PCA values smaller than zero) than in the remaining taxa (11 of 40 species). Notably, drought tolerance was shared with *S. lepidophylla*, the sister species of subgenus *Tetragonostachys*, and the only ecological niche shift supported by OU models was the one associated with the origin of the *Tetragonostachys* + *S. lepidophylla* clade.

In the PCA focused on subgenus *Tetragonostachys* (Appendix S4), the first two eigenaxes reflected gradients of temperature (TEMP; 39% variance explained) and precipitation (PREC; 27% variance explained). Although most species occurred in hot and arid habitats (Appendix S9), several *Tetragonostachys* species occurred in temperate-humid habitats (e.g., *S. deflexa*, *S. indica*, and *S. oregana*) or dry-cold tundra (*S. sibirica*). To further investigate the putative evolution of ecological niches within subgenus *Tetragonostachys*, we focused on the clade X because it provided a representative subset where niche evolution could be investigated along a highly supported phylogeny (Fig. 5). The available ecological data for this lineage ranged between 6 (*S. vardei*) and 639 (*S. rupestris*) specimens, with an average of 184 specimens per species. Only 3 species were represented by fewer than 50 specimens: *S. vardei* (six specimens), *S. steyermarkii* (13 specimens), and *S. shakotanensis* (14 specimens).

The TEMP eigenaxis revealed that a clade of six species (i.e., *S. densa*, *S. rupestris*, *S. watsonii*, *S. shakotanensis*, *S. vardei*, and *S. sibirica*) experienced a drastic ecological niche shift, corresponding to a migration from the Southwestern deserts to colder boreal and alpine environments (Fig. 5A, B). This ecological shift was highly supported by an OU model. Several comparable shifts were observed in earlier-diverging *Tetragonostachys* species (e.g., *S. leucobryoides*, *S. mutica*, *S. tortipila*, *S. underwoodii*, and *S. weatherbiana*; Appendix S9) but could not be included in the present analysis, owing to insufficient phylogenetic resolution. No clear patterns were observed for the PREC eigenaxis, where species were scattered across the ecological space without clear relation to the phylogeny. Accordingly, OU models did not support an ecological shift for the six aforementioned species along the PREC eigenaxis.

DISCUSSION

Our analyses revealed subgenus *Tetragonostachys* to be a highly supported clade (Fig. 1A), thereby confirming the results of previous studies that relied on analyses of only four species of the subgenus with *rbcL* (Korall and Kenrick, 2002, and references therein). Selaginellaceae are an ancient group, with a fossil history extending into the Carboniferous (Thomas, 1992, 1997). The dating analyses yielded a minimum crown-group age of *Selaginella* of 312–322 Ma, almost equivalent to the age of the fossil species *S. suissei*. Because the fossil most likely belonged to the stem group of most extant species (except *S. selaginoides* and *S. deflexa*), this age is likely an underestimate and may be closer to the early end of the confidence interval (321 Ma). The split between clades A and B was at 248, 279, and 281 mya (as estimated by PATHd8, BEAST, and r8s, respectively), which is in line with the observations of Korall and Kenrick (2002, 2004). The crown age of clade A was consistent across dating algorithms (240, 227, and 239 mya, as estimated by r8s, PATHd8, and BEAST, respectively) and may coincide with the separation of Laurasia and Gondwana (i.e., Late Triassic, 200 mya; Olsen, 1997). These results suggest that the *Tetragonostachys*

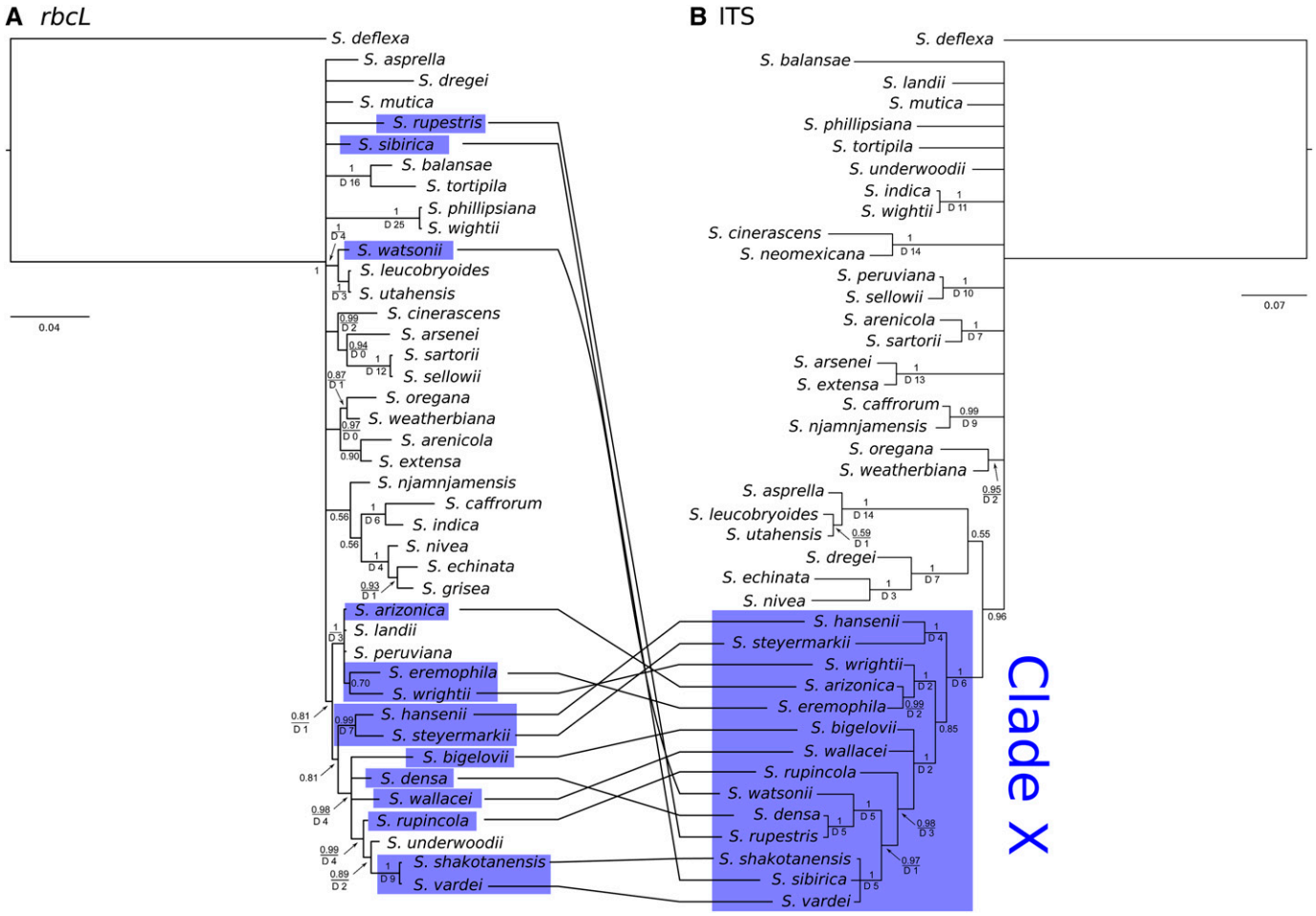


Fig. 2. Phylogeny of subgenus *Tetragonostachys* as revealed by (A) *rbcL* and (B) ITS. ITS notably resolves a well-supported clade "X" to which niche evolution models were applied (Fig. 5). Phylogenies are allcompat consensus trees inferred by MrBayes. For each node, Bayes posterior probabilities >0.5 appear above the horizontal line, and decay indexes supported by parsimony ratchet (with "D" as suffix) below the line. Nodes without decay indexes were not confirmed by parsimony ratchet; conversely, "D 0" outlines nodes confirmed by parsimony ratchet but showing no support. Topological incongruences among *rbcL* and ITS phylogenies involving members of that clade are identified by names highlighted in blue rectangles and connected by lines. Phylogenies are Bayesian allcompat consensus trees.

species, as well as *S. lepidophylla*, may have resulted from a vicariance event. This hypothesis would be consistent with the prevalence of central and North American species in the subgenus *Tetragonostachys*. Our dating analyses suggested either an Early Cretaceous (100–108 mya; BEAST and r8s) or a late Paleocene origin (59 mya; PATHd8) for subgenus *Tetragonostachys*. Even though the estimation of divergence times within the subgenus yielded large confidence intervals, our results showed that the subgenus had diversified during the Early Cretaceous–late Paleocene interval. This result is in line with other molecular

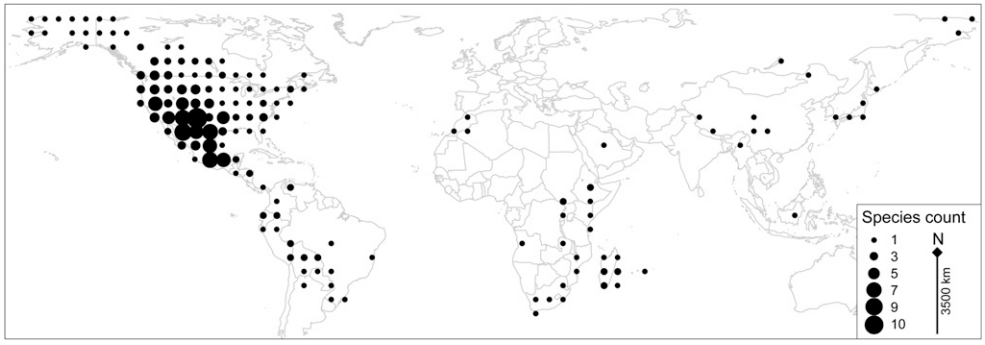


Fig. 3. Species diversity of subgenus *Tetragonostachys*, computed as species counts over a five-decimal degree resolution grid.

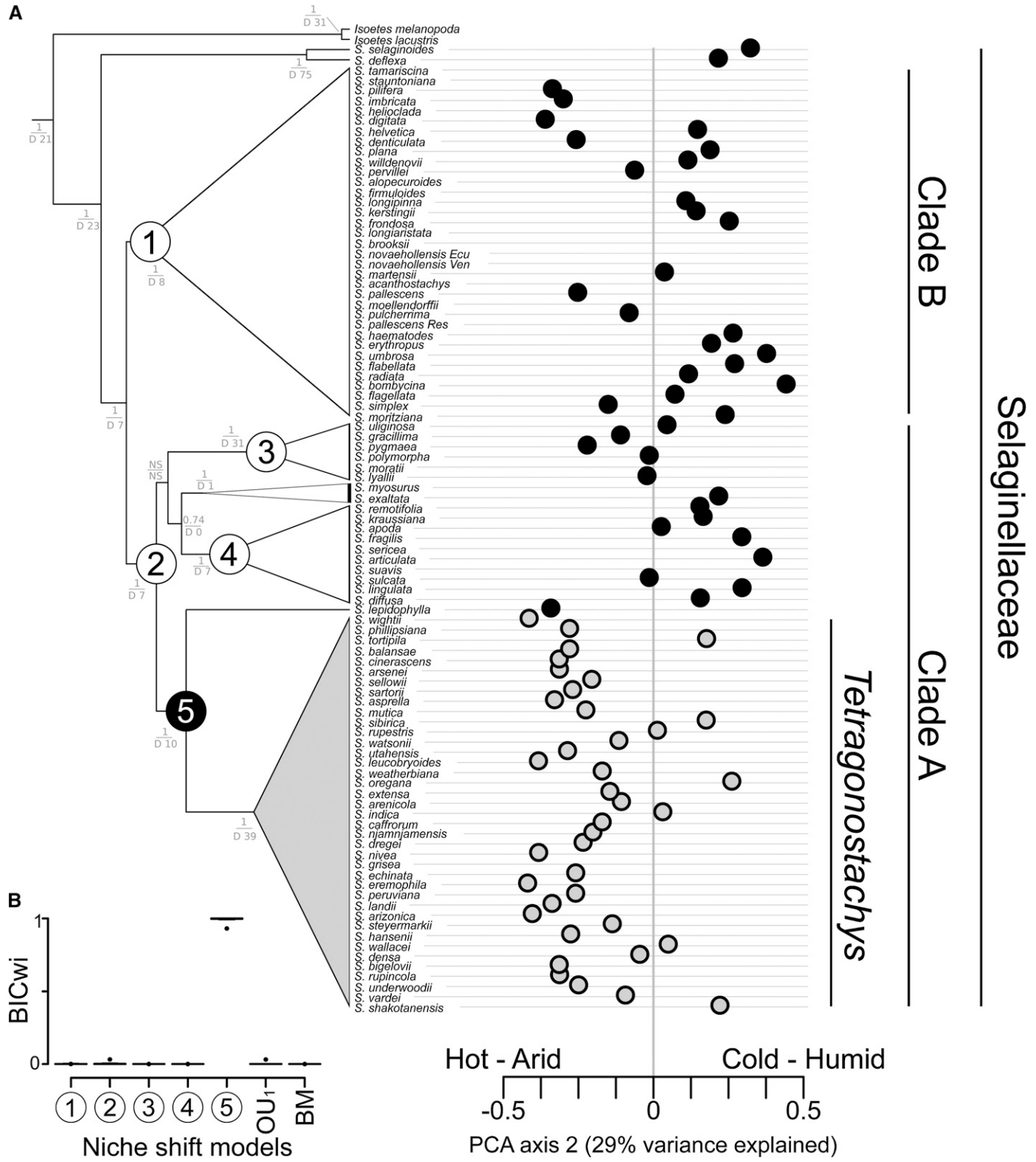


Fig. 4. Climatic niche evolution in *Selaginella*. (A) Genus phylogeny (allcompat *rbcL* Bayesian chronogram, dated using penalized likelihood) and corresponding species habitats, as described by a principal component eigenaxis (29% of the total variance is explained) discriminating hot and/or arid from cold and/or humid climatic niches. Niche-shift models considered median species values (represented here as dots). Nodes tested for niche shift significance are numbered 1–5 on the tree. Only node 5 significantly supported an ecological niche shift. (B) Statistical support (weighted Bayesian information criterion) of corresponding niche evolution models, assessing support for ecological shifts (nodes 1 to 5) vs. models assuming a unique ecological optimum for the whole tree (OU_1) or pure Brownian motion evolution (BM).

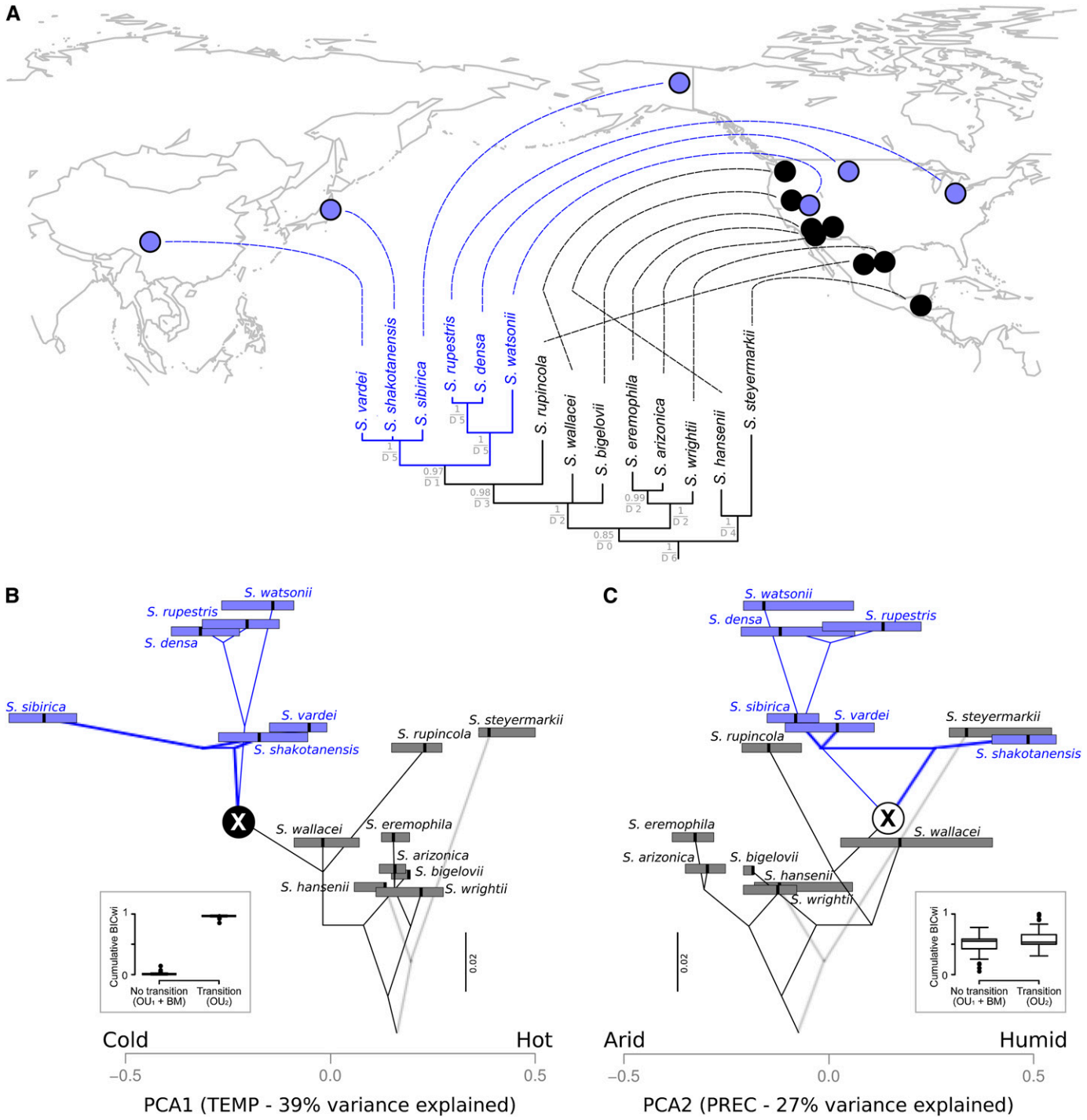


Fig. 5. Geographic distribution and niche evolution of the highly supported clade X. (A) ITS phylogeny (inset from Fig. 2) and corresponding geographic distribution of clade X species. The displayed geographic distributions are species centroids computed from herbarium vouchers. (B and C) Evolution of ecological niches, as supported by the ITS gene tree. Genetic distances and ecological niches are displayed on vertical and horizontal axes, respectively. Ecological variation observed within extant species is displayed as boxplots (25%, median, and 75% quartiles), ancestral ecological niches are inferred using the PIC method. Species with a significant ecological niche shift are highlighted in blue. The statistical support of ecological niche shift (weighted Bayesian information criterion) is assessed by comparing models considering a niche transition (OU_2 = model with two optima) to null-hypothesis models (sum of the weighted BIC of OU model with unique optimum and pure Brownian motion model).

phylogenetic studies investigating the extant diversity of lycophytes (Wikström, 2001).

From a biogeographic perspective, herbarium records revealed that most *Tetragonostachys* species occur in North and Central America (Fig. 1 B), supporting this region as the probable origin of the subgenus. Furthermore, during the estimated time frame of diversification, the North American continent was divided by the "Western Interior Seaway" (Kauffman, 1984). This would

support the high number of *Tetragonostachys* taxa found along the West Coast and the southwestern deserts of North America (Fig. 3) and the relative dearth of species on the eastern portion of the continent. Several members of the subgenus are endemic to Asia, Africa, or Madagascar, and two species (e.g., *S. sellowii* and *S. peruviana*) have ranges spanning North and South America. It is unclear whether the distributions of these species reflect vicariance or more recent dispersal events. Both *rbcL* and ITS supported a North American origin for two Asian species (*S. shakotanensis* and *S. vardei*), a pattern congruent with the current distribution of *S. selaginoides* (Heusser and Igarashi, 1994) and observations of recurrent plant and animal migrations across the Bering Strait (e.g., Fiorillo, 2008).

The early evolution of *Tetragonostachys* remains unclear, with a diversity of species and small clades arising from a basal polytomy. The basal polytomy appeared in both sequenced regions and is not directly attributable to technical limitations such as a lack of polymorphism or mutational saturation. Indeed, many shallow phylogenetic nodes are resolved with high decay indexes in the *rbcL* phylogeny, and detailed inspection of evolutionary distances showed limited effects of saturation for ITS (Appendix S5). Similar shortcomings were outlined by previous studies that reported, for instance, high substitution rates in *Selaginella* species (Korall and Kenrick, 2004). However, no studies have addressed whether reticulate evolution could have blurred deep phylogenetic relationships in Selaginellaceae. Indeed, we observed incongruent topologies between our *rbcL* and ITS gene trees (Fig. 2), most notably for clade X, which is highly supported in ITS but has members scattered across the *rbcL* tree. Although limited genome sampling could explain incongruence between our nuclear and plastid phylogenies, several additional observations suggest that hybridization may, indeed, have occurred during the evolution of *Selaginella*. Putative interspecific hybrids have been reported in botanical surveys of *Selaginella* (Yatskievych and Windham, 2009); the recently released *S. moellendorfii* genome showed evidence of hybridization (Banks et al., 2011); and at least two species belonging to subgenus *Tetragonostachys* (*S.* × *neomexicana* and *S. rupincola*; Yatskievych and Windham, 2009) are suspected to be of hybrid origin. Further investigation, preferably using phylogenomic approaches, will be necessary to assess the evolutionary role of reticulate evolution in *Selaginella*.

Alternatively, the observed basal polytomy might reflect a rapid radiation early in the evolutionary history of subgenus *Tetragonostachys*. This would be consistent with the shift to more arid habitats that developed during the middle Eocene (38–58 mya) in western North America (Minnich et al., 2007). Most *Selaginella* species occur in primary tropical rainforests, but Korall and Kenrick (2004) showed that xeric habitats have been colonized by at least three independent lineages. Our survey of ecological niches confirmed the prevalence of drought tolerance in subgenus *Tetragonostachys* and revealed strong phylogenetic patterning for this trait (Fig. 4). These results provide evidence that a drastic ecological shift occurred just prior to the separation of subgenus *Tetragonostachys* from its sister species (*S. lepidophylla*), which may have facilitated an adaptive radiation into the increasingly dry habitats of western North America. The adaptive value of drought tolerance was further suggested by another secondary ecological shift involving two Asian species (*S. shakotanensis* and *S. vardei*) and four close North American relatives (*S. densa*, *S. rupestris*, *S. sibirica*, and *S. watsonii*). This ecological shift involved a transition to colder, northern habitats (Fig. 5 B). Importantly, this shift was not significantly associated with a change in precipitation regimes, which suggests that at least five of the six species kept tracking dry habitats while simultaneously increasing their tolerance to colder climates. The underlying machinery of drought tolerance in *Selaginella* was recently investigated using physiological, proteomic, and genomic approaches (Iturriaga et al., 2006; Wang et al., 2010). These studies emphasized the role of the ABA signaling pathway in the accumulation of sucrose and proline, which helps ensure osmolyte balance and membrane protection and the activation of antioxidant system upon dehydration. Wang et al. (2010) also published detailed studies of drought tolerance in *S. tamariscina* (a clade B species). In this case, resilience in the face of dehydration is achieved by down-regulating all major metabolic processes, an approach that has not been documented in any other land plant (including bryophytes, gymnosperms, and angiosperms). To what extent drought-resistance mechanisms differ among the independently derived, xeric-adapted lineages of *Selaginella* is a topic worthy of additional research.

Our results provide a phylogenetic and ecological context for ongoing research on *Selaginella*, with special emphasis on the drought-tolerant subgenus *Tetragonostachys*. This is both timely and desirable, given that *Selaginella* is emerging as a model taxon in evolutionary botany owing to its long evolutionary history and its small genome that is readily accessible to next-generation sequencing. Despite the limited phylogenetic resolution achieved so far, our study provides important insights regarding the geographic and temporal origins of subgenus *Tetragonostachys*, as well as its ecological evolution. These results demonstrate the need for further investigation using phylogenomic approaches to explore the genetic bases of the detected ecological shifts. Understanding the various mechanisms that contribute to extreme drought tolerance in subgenus *Tetragonostachys* could have substantial benefits for crop-improvement programs and for human society at large.

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