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Original Article

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Evolution of cold tolerance in the highly stress-tolerant samphires and relatives (Salicornieae: Amaranthaceae)

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ABSTRACT

Low temperature constitutes one of the main barriers to plant distributions, confining many clades to their ancestrally tropical biome. However, recent evidence suggests that transitions from tropical to temperate biomes may be more frequent than previously thought. Here, we study the evolution of cold and frost tolerance in the globally distributed and highly stress-tolerant Salicornieae (Salicornioideae, Amaranthaceae *s.l.*). We first generate a phylogenetic tree comprising almost all known species (85-90%), using newly generated (*n* = 106) and published nuclear-ribosomal and plastid sequences. Next, we use geographical occurrence data to document in which clades and geographical regions cold-tolerant species occur and reconstruct how cold tolerance evolved. Finally, we test for correlated evolution between frost tolerance and the annual life form. We find that frost tolerance has evolved independently in up to four Northern Hemisphere lineages but that annuals are no more likely to evolve frost tolerance than perennials, indicating the presence of different strategies for adapting to cold environments. Our findings add to mounting evidence for multiple independent out-of-the-tropics transitions among close relatives of flowering plants and raise new questions about the ecological and physiological mechanism(s) of adaptation to low temperatures in Salicornieae.

Keywords: annuals; biome transition; distribution; frost tolerance; life history; niche evolution; temperature

INTRODUCTION

Adaptation to cold temperatures represents one of the most noteworthy instances of parallel evolution in the history of life on Earth. Adapting to the cold is complex, requiring a suite of physiological changes, and it is widely held that this limits the expansion of many clades into cold areas (Woodward 1990, Wiens and Donoghue 2004, Donoghue 2008, Preston and Sandve 2013, Zanne *et al.* 2018, Lancaster and Humphreys 2020). Accordingly, historical shifts between biomes, such as tropical-to-temperate shifts, are thought to have been rare (Wiens and Donoghue 2004, Crisp *et al.* 2009, Wiens *et al.* 2010). This is true also for angiosperms, which originated in the tropics, are much more diverse in tropical compared to temperate regions (Ricklefs and Renner 1994, Kreft and Jetz 2007, Kerkhoff *et al.* 2014) and which are considered unlikely to evolve adaptations to cold temperatures (on average <10°C), (Wiens and Donoghue 2004, Zanne *et al.* 2014, Nievola *et al.* 2017).

However, ongoing research is revealing more and more historical transitions between biomes, including out-of-the-tropics transitions, and even multiple transitions within single families [e.g. Poaceae (Edwards and Smith 2010, Schubert *et al.* 2020) and Malvaceae (Zizka *et al.* 2020a)] and genera (e.g. *Viburnum* L., Spriggs *et al.* 2015). In fact, relatively few clades have been studied in the detail required to detect multiple independent biome transitions among close relatives. It is therefore possible that biome transitions have been more frequent than generally assumed. In turn, this would imply that the complexities involved in adapting to new biomes, cold ones in particular, are less of a challenge for some groups than others. Here, we investigate a group of plants not previously studied for cold tolerance evolution, Salicornieae

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(Amaranthaceae *s.l.*), and relate our findings to the emerging patterns of more historical biome shifts than previously thought.

Salicornieae are one of four tribes currently classified in subfamily Salicornioideae (Morales-Briones *et al.* 2021) in Amaranthaceae *s.l.* (APG IV 2016; including the former Chenopodiaceae). Salicornieae comprise *c.* 100 species (Piirainen *et al.* 2017), some of which are referred to as samphires (notably species of *Salicornia* L. and *Tecticornia* Hook.f.). All species are succulent halophytes found in saline inland or coastal communities worldwide. Salicornieae are thought to have evolved from a common halophyte ancestor in Asia and the Mediterranean Basin at the Eocene–Oligocene boundary, and to have radiated into the main present-day lineages in the mid-Oligocene (Kadereit *et al.* 2006, Kadereit *et al.* 2012a, Piirainen *et al.* 2017). At that time, the climate was significantly warmer than today, but started to gradually cool and become drier by the mid-Miocene (Zachos *et al.* 2001, Mudelsee *et al.* 2014). Piirainen *et al.* (2017) proposed that this cooling and drying of the climate caused expansion of halophytic environments, in turn allowing the evolution and spread of halophytic plants; indeed, they found evidence of multiple dispersal events since the onset of the climatic cooling. Like most contemporary angiosperm lineages (Jansson *et al.* 2013, Zanne *et al.* 2014), Salicornieae thus originated in warm or even tropical areas, meaning the lineages in cold temperate or boreal areas today must have evolved adaptations to cold climates during their evolutionary history.

Salicornieae have been extensively studied due to their halophyte ecology and distinctive morphological, anatomical and physiological specializations. Thus, the biogeography, physiology and evolution of the clade are relatively well known (Piirainen *et al.* 2017). Additionally, with certain agricultural areas becoming increasingly saline, the potential for members of *Salicornia* to be used as crop plants is generating increased research interest (Singh *et al.* 2014, Bianciotto *et al.* 2016, Loconsole *et al.* 2019). Despite this attention, evolution of cold tolerance remains poorly studied in Salicornieae, and studies on cold tolerance in Amaranthaceae as a whole appear primarily focused on crop survival (Walker and Lutts 2014, Jalilian *et al.* 2017). In Amaranthaceae *s.l.*, Salicornieae provide a good starting point for studies of cold tolerance evolution because multiple genera are found in cold areas (Kadereit *et al.* 2006). Notably, the globally distributed *Salicornia* appears to be nested in a group of mainly Mediterranean, subtropical and tropical genera and to be phylogenetically distant from most other apparently cold-tolerant taxa (found mostly in *Halocnemum* M.Bieb., *Halopeplis* Bunge, *Halostachys* C.A.Mey. and *Kalidium* Moq.; Kadereit *et al.* 2006). This suggests either multiple gains or multiple losses of cold tolerance throughout the evolutionary history of Salicornieae. The special ecology and physiology also mean it is radically different from other angiosperm groups in which cold tolerance has previously been studied comparatively (e.g. Danthonioideae, Humphreys and Linder 2013, Pooideae, Schubert *et al.* 2019a; *Hypericum* L., Nürk *et al.* 2015 and *Viburnum*, Spriggs *et al.* 2015).

The monophyly of Salicornieae is not in question (Kadereit *et al.* 2006, 2012a, Kapralov *et al.* 2006, Piirainen *et al.* 2017). However, the clades within the tribe have been hard to distinguish due to their peculiar morphology, and the phylogenetic tree has been updated many times as first morphological and then genetic data have accumulated (see review in Piirainen *et al.* 2017). The latest full phylogenetic study (Piirainen *et al.* 2017) is based on data from four DNA regions [the external and internal transcribed spacers (ETS and ITS, respectively), and the *atpB-rbcL* and *matK-trnK* introns] for 67 taxa (58 species, nine subspecies). It recognizes *c.* 100 species belonging to 11 genera, with a 12th genus published simultaneously (Ball *et al.* 2017). Of the 12 genera all but two are small, comprising fewer than 10 species. The remaining two, *Salicornia* and *Tecticornia*, make up roughly three-quarters of the species richness, having both grown significantly in species number in the last 15 years, as previously recognized genera have been incorporated into them. *Salicornia*, including the paraphyletic *Sarcocornia*, is divided into four subgenera: *Afrocornia* Piirainen & G.Kadereit; *Amerocornia* Piirainen & G.Kadereit; *Arthrocnemoides* Ung.-Sternb.; and *Salicornia*, distributed across all continents except Antarctica (Piirainen *et al.* 2017). Meanwhile, *Tecticornia*, with the incorporation of *Halosarcia* Paul G.Wilson, *Pachycornia* Hook.f., *Sclerostegia* Paul G.Wilson; and *Tegicornia* Paul G. Wilson (Shepherd and Wilson 2007), is an almost exclusively Australian genus.

Support for the monophyly of each of the 12 genera is strong, but the phylogenetic structure within and among the genera is less clear. The small genera and *Salicornia* have been comprehensively sampled by Piirainen *et al.* (2017) and Ball *et al.* (2017), with only a few missing species, but *Tecticornia* was comparatively undersampled in these studies. Molecular phylogenetic trees of *Tecticornia* exist (Shepherd *et al.* 2004), but are based on a partly different set of markers. The first goal of this study is therefore to pool all existing DNA sequence data, add previously unsampled taxa and increase data coverage for already sampled taxa. The combined data will be used to produce a well-resolved phylogenetic tree for the entire tribe, with more comprehensive taxon sampling than previous estimates. This will provide the basis for a rigorous tribe-wide analysis of cold tolerance evolution.

The next goal is to study cold tolerance evolution using phylogenetic comparative methods. We use large-scale geographical occurrence and climate data to construct approximations of the realized climatic niches of species. The optimal growth range for most plants is between 10 and 30°C, and chilling stress can occur anywhere between 0 and 15°C (Nievola *et al.* 2017, Schubert *et al.* 2020). Thus, cold tolerance is here defined as occurrence in cold areas, which to most plants means those that experience temperatures <10°C (Nievola *et al.* 2017). By definition, therefore, there is no explicit link to physiology, nor is a distinction made between different cold tolerance strategies (e.g. escape, avoidance, resistance, or true tolerance; Sakai and Larcher 1987). This approach has been shown to approximate the fundamental niche (at least on a relative scale; e.g. Humphreys and Linder 2013). Further, because freezing temperatures are particularly challenging for many organisms (Sakai and Larcher 1987, Woodward 1990), we also specifically study which species occur in freezing areas (minimum temperatures <0°C according to the BioClim variable BIO6; Fick and Hijmans 2017; see Methods). We reconstruct ancestral states to test how many times cold tolerance has evolved independently, and provide a first test of which traits may constitute, or be associated with, cold and frost adaptations by testing for correlated evolution between frost tolerance and life history strategy.

Charnov and Schaffer (1973) showed that an annual life history is more beneficial if the rate of adult survival is low compared to the number of offspring. This has been used to suggest that annuals should be more prevalent in hostile environments, e.g. those experiencing high levels of seasonal variation or disturbance (Charnov and Schaffer 1973, Humphreys *et al.* 2011, Hjertaas *et al.* 2022), something that has been indicated in several clades, including Poaceae (Verboom *et al.* 2003), Onagraceae (Evans *et al.* 2005), Scrophulariaceae (Datson *et al.* 2008), and Saxifragaceae (Conti *et al.* 1999). In seasonally frozen areas, annuality may be adaptive by allowing escape from the worst winter temperatures, or, because annuals tend to grow fast, by enabling them to complete their life cycles during short growing seasons. However, annuals do not tend to dominate cold areas as they might warm ones (Körner 2003), so demonstrating a correlation between the annual life history strategy and occurrence in freezing areas would be particularly interesting. In Salicornieae, in which annuals have evolved multiple times (Piirainen *et al.* 2017), there is some evidence suggesting such a correlation. Kadereit *et al.* (2007) noted that the exclusively annual *Salicornia* subgenus *Salicornia*, in which the annual life form probably evolved sometime in the last 10 million years (Myr; Kadereit *et al.* 2006, Piirainen *et al.* 2017), grows much further north than the rest of *Salicornia.* They suggested annuality could be a key innovation for inhabiting areas with 'severe winter frosts' and hypothesized that it might have evolved as an adaptation to increasingly 'frosty seasons'. Alternatively, they suggested annuality may have evolved in a subtropical lineage where it enabled colonization of seasonally flooded areas, and only later facilitated establishment in frosty areas. The first scenario thus suggests the environment (frost) came first, followed by the trait (annuality), whereas the second suggests the reverse. Meanwhile, the annual life history strategy is absent from other high-latitude taxa such as *Kalidium* and cannot have facilitated their adaptation to cold. Support for correlated evolution between life history and freezing tolerance will increase understanding of the evolution of Salicornieae regardless of the nature of such a correlation. Furthermore, disentangling whether low temperature tolerance or annuality evolved first will indicate whether annuality served as an adaptation to, or a prerequisite for, inhabiting freezing areas.

This study thus has four principal aims: (i) to provide an improved species-level phylogenetic tree of Salicornieae; (ii) to document in which clades and geographical regions cold and frost-tolerant species occur; (iii) to test whether cold tolerance has evolved once or multiple times; and (iv) to test whether the evolution of frost tolerance and life history (annual/perennial) are dependent and, if so, which trait evolved first.

MATERIAL AND METHODS

Taxon sampling and genetic data

To maximize species sampling across the tribe, available published and unpublished DNA sequence data were collated for the most commonly used markers (ETS, ITS, *atpB-rbcL*, and *matK-trnK*). In addition, sampling gaps in available data were filled with newly generated sequences for a few taxa for which suitable plant material could be obtained. Sequences were downloaded for all Salicornieae species and subspecies currently published on GenBank. The sampling was limited to one accession per taxon and marker and sought to maximize the number of accessions used from the same isolate. In cases where sampling from a single isolate was not possible, secondary isolates were chosen from the geographical area closest to the primary isolate wherever possible. Additionally, isolates used by Piirainen *et al.* (2017) or verified by Salicornieae experts were prioritized. *Allenrolfea* Kuntze showed signs of non-monophyletic species in Piirainen *et al.* (2017), with accessions of *A. vaginata* Kuntze nested in *A. occidentalis* Kuntze. Therefore, several accessions of each putative species were downloaded in an attempt to detail the phylogenetic structure of the genus and resolve the position of the previously unsampled *A. patagonica* Kuntze.

Outgroups were chosen to represent a broader sampling of Amaranthaceae, and include representatives of *Bassia* All., *Chenolea* Thunb., *Chenopodium* L., *Grubovia* Freitag & G.Kadereit, *Sclerolaena* R.Br., and *Suaeda* Forssk. Of these, all but *Chenopodium* and *Suaeda* are in Camphorosmeae (Kadereit and Freitag 2011). Suaedeae (including *Suaeda*) is the closest sister to Salicornieae, followed by Camphorosmeae. *Chenopodium* is the most distant outgroup, Chenopodioideae being sister to all other clades sampled here (Morales-Briones *et al.* 2021). *Bienertia* Bunge, a close relative to Salicornieae often included in phylogenetic analyses of the tribe (e.g. Shepherd *et al.* 2005, Kadereit *et al.* 2006, Kapralov *et al.* 2006), was not included here following reports of it producing conflicting topologies (Piirainen *et al.* 2017). A complete list of sequences used in the study is provided in Supporting Information, Table S1.

DNA extraction, amplification, sequencing, and alignment

New samples include herbarium specimens, existing silica-dried field samples, and some of the isolates and DNA extractions used by Piirainen *et al.* (2017); (Supporting Information, Table S2). For all samples except *Tecticornia* (see next paragraph), DNA extractions were performed using the DNeasy Plant Mini Kit (Qiagen®), following the manufacturer's instructions. The quality of the extractions was examined using NanoDrop™ (Thermo Scientific™, model: ND-ONE-W). Polymerase chain reaction (PCR) amplifications were done in the VWR® UNO cycler 732-1200 and the Applied Biosystems™ GeneAmp PCR System 9700 (see Supporting Information, Tables S3 and S4 for programmes and primers; programmes were chosen to maximize yield following experimentation). Products were visualized on 1% agarose gels and purified using ExoSAP-IT™ PCR Product Cleanup Reagent (Thermo Fisher Scientific Inc.). Sequencing was performed by Macrogen Europe (Amsterdam, The Netherlands) following their EZ-Seq protocol. Contigs were edited and combined using the Staden package (Staden 1996), using PreGap to prepare files and Gap4 to assemble consensus sequences. In instances where no contiguous sequence could be found in Gap4, BioEdit (Hall 1999) was used to manually create and edit a consensus sequence.

For *Tecticornia* samples, extraction of DNA was carried out using the DNeasy Plant Mini Kit (Qiagen®) according to the manufacturer's instructions, with the following modifications: the lysis step was extended to 15 min, and the elution was carried out in two lots of 20 min for a combined volume of 200 μL. DNA

(10 μL) was separated on a 0.8% (w/v) agarose Tris-acetate-EDTA (TAE) gel to examine quality. PCR amplifications were done in a Biometra Tgradient Thermocycler (see Supporting Information, Tables S3 and S4 for programmes and primers). Products were visualized on a 0.8% (w/v) agarose TAE gel and purified using ExoSAP-IT™ PCR Product Cleanup Reagent (Thermo Fisher Scientific Inc.). Sequencing reactions were completed using the BigDye Terminator v.3.1 Cycle Sequencing Kit (Applied Biosystems), according to the manufacturer's instructions. Products were purified using an Illustra Sephadex G-50 Fine DNA Grade (GE Healthcare) column. Samples were loaded on an automated sequencer (16 capillary 3130x1 Genetic Analyser, Applied Biosystems). The resulting chromatograms were edited using Sequencher v.4.1.4 (Gene Codes Corporation) or Geneious v.6.1.7 (https://www.geneious.com).

All sequence alignments were done manually in Mesquite (Maddison and Maddison 2019), as alignment was generally unambiguous. Ends were trimmed to remove columns with >50% missing characters. Ten matrices were made: one for each separate marker (four matrices) and three combined matrices (just plastid markers, just ribosomal markers, and all four markers concatenated) for all accessions analysed. In addition, three reduced matrices pruned to include only one accession per species were generated (plastid DNA, nrDNA, and combined). Alignment summary statistics are presented in Supporting Information, Table S5.

Final species sampling

Not counting outgroups, sequence data for 366 accessions and 119 taxa were downloaded from GenBank. We contributed 106 newly generated sequences to the analysis, adding 44 taxa, and filling gaps in gene regions for another 11 (Supporting Information, Table S2). Sequencing of *Allenrolfea patagonica* Kuntze failed, and because of the limited number of *Allenrolfea vaginata* accessions available on GenBank, no additional inference about the infrageneric phylogenetic structure of *Allenrolfea* is possible in this study. In total, 122 taxa were included in the final matrices, representing 101 species and 21 subspecies. All genera were completely sampled except *Allenrolfea* (two of three species), *Arthrocaulon* Piirainen & G.Kadereit (one of two or three species), *Salicornia* (85–90% of species), and *Tecticornia* (86% of species).

Phylogenetic analysis

First, individual gene trees were generated and examined for conflict [supported differences with >70% bootstrap support or 0.95 Bayesian posterior probability (PP)]. Where conflict was found, conflicting taxa were duplicated following Pirie *et al.* (2008, 2009). We used this approach to avoid concatenating conflicting partitions, which can lead to information loss, decreased support and incorrect inferences in downstream analyses (Pirie *et al.* 2009). Four taxa were duplicated: *Arthroceras subterminale* (Parish) Piirainen & G.Kadereit; *Kalidium schrenkianum* Bunge ex Ung.-Sternb.; and both subspecies of *Microcnemum coralloides* Loscos & J.Pardo Font Quer. In the reduced matrix, pruned to include only one accession per species, duplicated taxa were also retained, although subspecies of *M. coralloides* were represented by a single accession.

Best-fitting substitution models were determined based on Akaike information criterion (AIC) using jModeltest v.2.1.10 (Darriba *et al.* 2012) on the CIPRES Science Gateway (Miller *et al.* 2010; Supporting Information, Table S6). Maximum likelihood (ML) analyses were run in RAxML (Stamatakis 2014), using default settings, and Bayesian analyses were run in MrBayes v.3.2.7a (Ronquist *et al.* 2012). The Bayesian analyses used four MCMC chains of one million generations, sampled every 1000 generations, for all datasets except the combined four-region matrices, which ran for three million generations, sampling every 3000 generations. For all analyses, a burn-in of 0.25 was used. All phylogenetic analyses were performed on the CIPRES Science Gateway. For analysis of concatenated matrices, partitions were unlinked and given separate models (Supporting Information, Table S6).

Molecular dating

Dating analyses for the reduced, four-region tree (one accession per species, apart from for duplicated taxa) were carried out in BEAST v.1.10.4 (Suchard *et al.* 2018). The main goal of this analysis was to time calibrate the tree as required by some downstream analyses, not to provide new estimates of node ages as there is little new evidence that could alter the result of Piirainen *et al.* (2017). Node ages were calibrated using the fossil *Salicornites massalongoi* Principi (Principi 1926) and two confidence intervals extracted from the analysis of Kadereit *et al.* (2012a). This yielded calibration points for crown Salicornieae (35.4–23.3 Myr), crown Salicornieae + Suaedeae (28.6–49.8 Myr) and at the crown of the newly expanded Salicornioideae (comprising Suaedeae, Salicornieae, Salsoleae, and Camphorosmeae, also referred to as 'Chenopods II'; 34.7–61.3 Myr; Morales-Briones *et al.* 2021). Input files were generated in BEAUTi v.1.10.4 (Suchard *et al.* 2018). Monophyly was constrained at the calibration points and for the outgroup *Chenopodium*, and age priors were lognormally distributed around the mean and standard deviation. Substitution models were set for each gene region separately (Supporting Information, Table S6) using a relaxed lognormal clock model applied across all trees and a birth-death tree model. The MCMC analysis was initiated independently twice on a random starting tree and run for 90 million iterations with a sampling frequency of 1000. Convergence was assessed using Tracer v.1.7.1 (Rambaut *et al.* 2018) as all parameters having an estimated sample size >200, and no transition kernels at 0 acceptance rate. The output was resampled in LogCombiner v.1.10.4 (Suchard *et al.* 2018) with a sampling rate of 10 000, and a consensus tree was assembled using TreeAnnotator v.1.10.4 (Suchard *et al.* 2018) with a burn-in of 25%.

As a final step, and prior to the trait evolution analyses, the dated, fully dichotomous tree was pruned to include only species with occurrence data and limit outgroups. Subspecies retained in the tree due to topological uncertainty were assigned identical trait values. Outgroups retained in the comparative analyses were chosen to represent the sister clade Suaedeae occupying extreme ends of the cold tolerance spectrum. *Suaeda corniculata* Bunge and *S. olufsenii* (Pauls) G.L.Chu, which occur in cold parts of Central Asia, were chosen to represent cold-tolerant taxa. *S. aegyptiaca* (Hasselq.) Zohary and *S. spicata* Moq. were picked to represent cold-sensitive taxa.

Climate and occurrence data

Geographical occurrence data were obtained from the Global Biodiversity Information Facility (GBIF 2020a, 2020b).

Occurrences were compiled using the GBIF Occurrence Download interface and accessed from R (R Core Team 2020) via the 'rgbif' package (Chamberlain and Boettiger 2017, Chamberlain and Oldoni 2020). Occurrences with a precision of fewer than two decimal points were removed, and the data were then filtered using 'CoordinateCleaner' (Zizka 2020). Filtering removed coordinates in seas and close to capital cities, country centroids, biological institutions, and the GBIF-headquarters and coordinates that did not match the country code reported for that record, reported as zeros or with equal values for longitude and latitude. All settings for the filtering were kept as default following close inspection of the occurrences lost in each step. In total, 96 407 georeferenced observations for Salicornioideae were downloaded, and after filtering and reduction to one observation per species per grid 24 914 unique occurrence localities remained. Temperature data were downloaded from the WorldClim database (Fick and Hijmans 2017) and variables BIO3 (isothermality, a measure of seasonal relative to diurnal temperature variation), BIO6 (minimum temperature of the coldest month), and BIO11 (mean temperature of the coldest quarter) were extracted (at 30 s resolution) for each unique georeferenced occurrence locality using 'raster' (Hijmans 2020).

Cold tolerance evolution can be challenging to study because of the difficulty of defining at what temperature organisms experience cold stress. Given that cold sensitivity differs among species and may vary under different conditions (e.g. at different levels of moisture), we reconstructed ancestral states on a continuous scale, rather than give a specific temperature limit. However, because freezing poses separate challenges that require a suite of different physiological responses, freezing tolerance was also analysed by treating the data as a binary variable. Thus, two datasets were generated. The first treated cold tolerance as a continuous trait, represented by the average BIO3, BIO6, and BIO11 values for a species (Supporting Information, S1). Estimates of species means from fewer than 10 geographical occurrence records were considered unreliable, and thus 23 species were removed from this dataset. In the second dataset, frost tolerance was coded as a binary trait, based on whether the mean for BIO6 for a species was <0°C or not (Supporting Information, S2). Species with fewer than 10 occurrences were kept in this dataset if they could be confidently coded as experiencing frost or not.

Cold tolerance evolution

To create the continuous variable of cold tolerance, means and confidence intervals of each BioClim variable (BIO3, BIO6, and BIO11) were computed for each species. To determine whether species means were sufficient to describe the variation in cold tolerance, the correlation between the mean, minimum (95%) and maximum (95%) values was assessed using Kendall's Tau. Pairwise comparison found strong correlations between mean, minimum, and maximum values of all three BioClim variables (Supporting Information, Table S7); thus, we proceeded by analysing species means.

To test for patterns in the evolution of cold tolerance, three models of trait evolution were fitted for each BioClim variable using the 'transform.phylo.ML' function in 'motmot' (Puttick *et al.* 2019). Models were Brownian motion (BM) and BM with branch length transformation parameters lambda and delta (Pagel 1999). Models were compared using AICc. Ancestral state reconstruction was performed with the 'ace' function in 'ape' (Paradis and Schliep 2019), by transforming the branch lengths according to the best-fitting model using the 'rescale' function in 'geiger' (Pennell *et al.* 2014) and then estimating ancestral states on the transformed trees using BM.

Frost tolerance evolution

The evolution of frost tolerance was modelled using the 'hidden rates model' in 'corHMM' (Beaulieu *et al.* 2021). The hidden rates method allows modelling the evolution of a binary trait with different transition rates in different parts of the tree, using MCMC to fit a model with a specified number of rates (Beaulieu *et al.* 2013). If a model with more than one rate is deemed to have the best fit, the trait is inferred to change more rapidly on some branches than others. This allows the construction of a more accurate model than if all rates are forced to be the same. Models were fitted with up to five rates (the maximum permitted in corHMM), each run with 100 random restarts, and model fit was assessed using AICc.

The effect of an annual life history on frost tolerance evolution

First, the evolution of life history was modelled using the 'hidden rates model', as for frost tolerance. This was done to visualize the best-fitting ancestral state reconstruction for life history in the context frost tolerance evolution but without them being part of the same model. Next, to test whether frost tolerance evolution is correlated with life history evolution, Pagel's model of correlated evolution was used (Pagel 1994). Two models were fitted using the Discrete function in BayesTraits v.3.0.2 (available from www.evolution.reading.ac.uk): one where the traits were allowed to evolve independently (meaning that transition rates in the first trait have the same rate irrespective of the state of the second trait; four rate parameters) and one where they were dependent (meaning that transition rates of one trait may differ depending on the state of the second trait; eight rate parameters; Supporting Information, Table S8). Models were fitted using both ML (1000 ML tries) and MCMC (Pagel *et al.* 2004), with chains run for 50 million generations from a random starting tree. MCMC output was assessed by acceptance rate (which ranged 0.30–0.35 and was therefore deemed appropriate) and the log likelihood was sampled using the BayesTraits stepping stone sampler (1000 stones, 10 000 iterations each). ML models were compared using the likelihood ratio test, and Bayesian models using Bayes factors.

MCMC runs were initially conducted with default priors (uniform distribution, sampling between 0 and 100), but model diagnostics showed a lack of convergence for parameters in the dependent model. Thus, the ML parameter estimates were used as guides to assign new priors. All parameters that failed to converge were given gamma-distributed priors with means and variances picked from a uniform distribution between 0 and 1 using the Hyperprior option. The q24 and q42 parameters showed no convergence problems and thus their default priors were retained. Convergence was assessed visually, ensuring that the posterior likelihoods showed no trends or anomalies.

The models of correlated evolution used above have been shown to have high type I error rates (i.e. rejection of a null hypothesis that is true; e.g. Maddison and Fitzjohn 2015, Rabosky and Goldberg 2015). We therefore repeated these analyses on data simulated to represent a binary trait (annual/perennial) that has evolved independently of frost tolerance. Thus, we expect that the null (independent) model should not be rejected in favour of the alternative (dependent) model for simulated data. If it is, then the difference in fit needs to be smaller for simulated data than for our observed (empirical) data, for any observed correlation to remain statistically significant. We fitted the independent and dependent models to each simulated trait using ML analysis and compared their fit using likelihood ratios (LRs). Finally, we compared the LR difference in fit between the independent and dependent models across simulated traits to the LR difference in fit for our empirical data. Data were simulated by generating 1000 continuous traits under BM on our observed phylogenetic tree and then converting each to a binary trait using an arbitrary threshold, where the lowest 17 values were scored as annual and the highest 87 values as perennial (representing the proportion of each state in our observed life history trait). Data were simulated using 'bmPlot' in Phytools v.0.7-70 (Revell *et al.* 2012).

Sensitivity analyses (coding frost tolerance using species minima instead of means)

Finally, to test for robustness of results to variation in definitions of frost tolerance, the corHMM and BayesTraits analyses were rerun with frost tolerance coded using the lower 95% confidence interval (CI) for BIO6 rather than the mean (i.e. whether the lower 95% CI for a species indicated it occurred in freezing areas or not). This resulted in an additional 11 species being scored as frost tolerant (*Allenrolfea occidentalis*, *Microcnemum coralloides*, *Salicornia alpini* Lag., *Salicornia ambigua* Michx., *Salicornia andina* Phil., *Salicornia bigelovii* Torr., *Salicornia patula* Duval-Jouve, *Salicornia depressa* Standl., *Salicornia procumbens* Sm., *Salicornia ramosissima* J.Woods and *Suaeda spicata*).

RESULTS

Phylogenetic analyses and molecular dating

The full, four-partition Bayesian tree closely resembles the tree produced by Piirainen *et al.* (2017); Salicornieae are monophyletic (PP = 1), as are all multispecies genera except *Tecticornia* (PP < 0.9), which remains unresolved (Fig. 1; see Supporting Information, Figs S1–S6 for individual gene trees, nrDNA, and plastid DNA trees). *Salicornia* is subdivided into four distinct clades, designated by Piirainen *et al.* (2017) as subgenera *Salicornia* (PP = 1), *Amerocornia* (PP = 1), *Arthrocnemoides* (PP = 1), and *Afrocornia* (PP < 0.9). They represent the former *Salicornia* and the American, European, and South African/Australian clades of the former *Sarcocornia* A.J.Scott. Among the other lineages, the three main clades of Salicornieae receive support, and the deep five-way polytomy found by Piirainen *et al.* (2017) is partly resolved. The American clade comprising *Allenrolfea* and *Heterostachys* Ung.-Sternb. (PP = 1) diverged first, with those two genera being sister to the rest of Salicornieae (PP = 0.96). The resolution within the two other major clades (both PP = 1) remains limited, the sister relationship of *Halostachys* and *Halocnemum* being the supported clade (PP = 1). *Arthrocaulon* appears to be sister to the *Tecticornia* and *Salicornia* clade (PP = 0.97), but the conflicting positions of *Arthroceras* Piirainen & G.Kadereit and *Microcnemum* complicate the inference of relationships among those genera. Finally, the dated tree is similar to that of Piirainen *et al.* (2017; Supporting Information, Fig. S7).

Occurrence of Salicornieae in cold and freezing areas

The species experiencing the coldest winters are mostly found in *Salicornia*, *Halocnemum*, *Halopeplis*, *Halostachys*, and *Kalidium* (Fig. 2). For all these genera, occurrences in cold areas are mainly confined to the Northern Hemisphere, *Salicornia* being widespread and occurring in colder parts of North America and Eurasia, whereas the remaining genera are confined to Central Asia, the Middle East and Africa.

Cold tolerance evolution

Pagel's λ is the best model (ΔAICc > 120, Table 1) and was used to transform branches for ancestral state reconstruction. CIs of λ are close to 1 ($0.70 < \lambda < 0.96$), indicating models similar to BM, where most trait variance correlates with phylogeny. The models found here deviate from BM in that more evolutionary change is inferred to have occurred towards the tips of the tree.

Visual inspection of the ancestral state reconstructions (Fig. 3) suggests the root of Salicornieae may have been moderately cold tolerant, and that this tolerance has then increased in the *Kalidium-Halocnemum-Halopeplis-Halostachys* clade, been lost in most other lineages, but retained or re-evolved in *Salicornia*. CIs (95%) for values at the root of Salicornieae indicate an isothermality (BIO3) of 33.1–51.0%; a minimum temperature of the coldest month (BIO6) of −8.69 –+6.81°C; and a mean temperature of the coldest quarter (BIO11) of −1.63–+13.5°C. For all three variables, there are species with means both below and above the root interval (Supporting Information, Supplementary Data, S1), and for both BIO6 and BIO11 there are more species above the root interval than below it (25 vs. nine for BIO6 and 23 vs. nine for BIO11).

Frost tolerance evolution

Frost-tolerant species are found in the *Kalidium-Halocnemum-Halopeplis-Halostachys* clade and in *Salicornia* (Fig. 4). Inspection of the modelled hidden rates show that the two-rate model has the best fit (LL = -34.9, ΔAICc > 13, Supporting Information, Table S9), and that it is possible to distinguish a fast and a slow rate of frost tolerance evolution (Table 2). The slow rate (in any state) is more common and found in all major clades. Most frost-sensitive lineages are inferred to be in the slow rate category. The fast rate, on the other hand, appears to be confined to and prevalent on the branches where frost tolerance occurs, although it is notably absent from *Kalidium*. Frost-tolerant lineages are thus found in either the fast (*Salicornia* and *Halocnemum-Halopeplis-Halostachys*) or slow (*Kalidium*) rate category, indicating the existence of two different types of frost tolerance in Salicornieae. However, the state at most deeper nodes is not reconstructed with certainty. Accordingly, the probability that the common ancestor of Salicornieae was frost tolerant is estimated

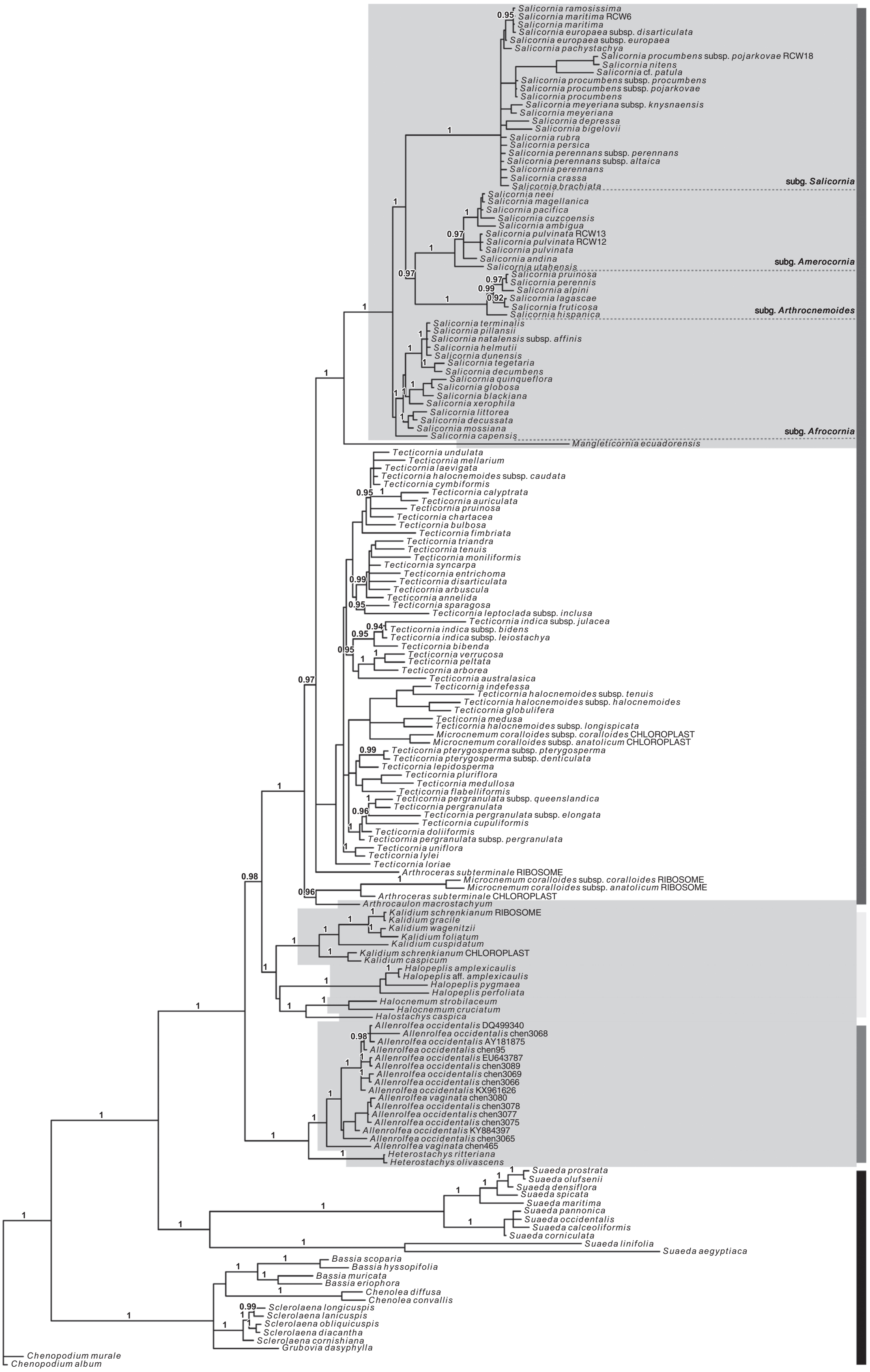

Figure 1. Bayesian phylogram of Salicornieae (Salicornioideae, Amaranthaceae) based on concatenated ETS, ITS, atpB-rbcL, and matK-trnK sequences. Numbers above branches correspond to posterior probabilities (PPs) and are displayed where PP > 0.90. Supported genera are highlighted in light grey, and the outgroups and three main clades of Salicornieae are marked with vertical bars. Additionally, Salicornia

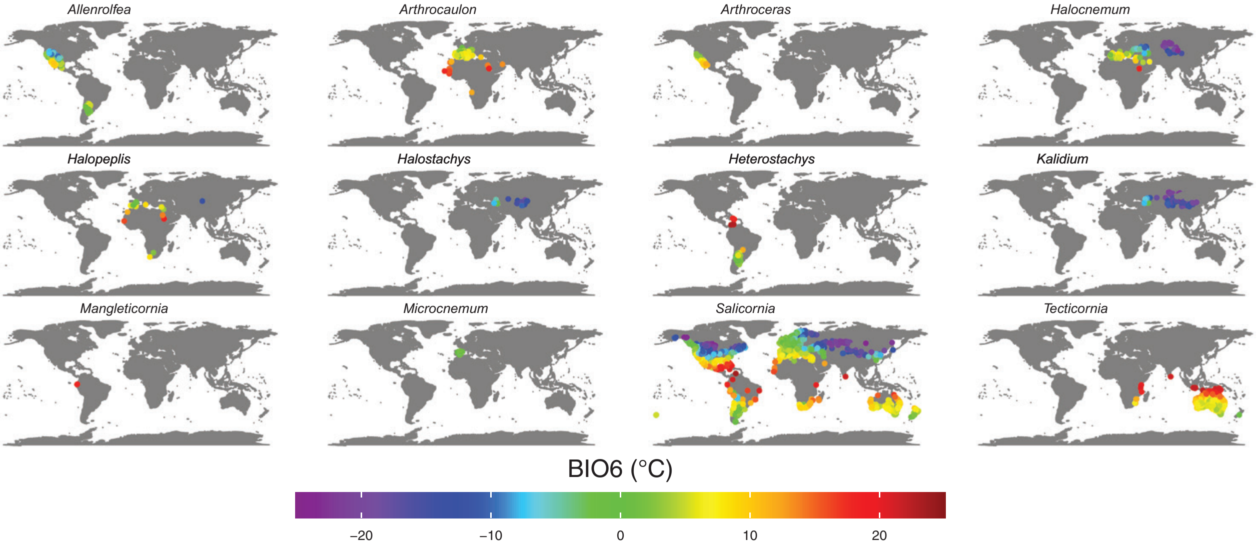

Figure 2. Geographical distribution of each genus of Salicornieae (Salicornioideae, Amaranthaceae). Dots represent filtered georeferenced occurrence points from GBIF and are coloured according to the minimum temperature of the coldest month [BIO6, minimum temperature of the coldest month (°C)] at each locality.

Table 1. Fit and parameter estimates of three models of cold tolerance evolution for the three BioClim variables studied.

	BM	λ	δ	λ[*]	δ[*]
	(LL, AICc)	(LL, AICc)	(LL, AICc)		
BIO3	−352, 708	**−275, 557**	−337, 681	0.87 (0.70–0.96)	5 (4.30–5)
BIO6	−361, 726	**−264, 534**	−346, 698	0.87 (0.70–0.96)	5 (4.35–5)
BIO11	−352, 709	**−259, 525**	−337, 681	0.88 (0.73–0.96)	5 (4.33–5)

BM = Brownian motion; λ and δ = Pagel's lambda and delta models; LL = log likelihood; AICc = sample-size corrected Akaike information criterion (AIC); BIO3 = Isothermality; BIO6 = Minimum temperature of the coldest month; BIO11 = Mean temperature of the coldest quarter. Best-fitting models are shown in bold.

[*] Values in brackets show 95% confidence intervals of estimates.

to be 0.25 (in either the fast or slow rate category), i.e. most probably frost sensitive but with considerable uncertainty.

Assuming that the clade was not ancestrally frost tolerant (the most likely reconstructed scenario), the reconstruction further suggests up to four independent origins of frost tolerance in Salicornieae: in the *Salicornia maritima* clade, the *Salicornia andina* clade, the *Halocnemum-Halopeplis-Halostachys* clade and in *Kalidium*. The last two cases might have a common origin but are now in different rate categories, indicating different types of frost tolerance. The first two cases (within *Salicornia*) represent two independent origins according to the reconstructed scenario. Their most recent common ancestor was most probably in the sensitive-slow category. This scenario is not reconstructed with absolute certainty, but is the most likely scenario modelled.

The effect of an annual life history on frost tolerance evolution

The hidden rates model with the best fit for life history was the single-rate model ($LL = -21.4$, $\Delta AICc > 7$, Supporting Information, Table S9), meaning that gains and losses of annuality are inferred to have occurred with the same rate throughout the tree. The ancestral states reconstructed using this model depicts annuals to be mostly absent from the deeper nodes of the tree (Fig. 4); the probability that the ancestor of Salicornieae was an annual is 0.03 and the annual life history is inferred with certainty only for internal nodes of the predominantly annual clades of *Halopeplis* and *Salicornia* subgenus *Salicornia*.

Based on visual inspection of the output of Pagel's correlation models, all models converged (Supporting Information, Fig. S8). The independent model was rejected in both the ML and MCMC analyses (ML: likelihood ratio test: $\chi^2 = 21.4$, d.f. = 4, $P < 0.001$; MCMC: logBF = 13.8), indicating that frost tolerance and life history have evolved dependently in this clade. However, the results of the analyses of simulated data also showed that the independent model was a worse fit to data than the dependent model for most of the simulated traits ($LR > 0$; Supporting Information, Fig. S9). Furthermore, our observed difference in fit between the independent and dependent models ($LR = 21.4$) lies within the 95% CI of the difference in fit across the simulated data. Thus, there is no signal in our data as a whole beyond that expected for two independently evolving traits; the null model of independent evolution of frost tolerance and life history cannot be rejected.

subgenera are labelled. For ease of visualization, the root branch of Chenopods II (all taxa except Chenopodium) has been shortened. CHLOROPLAST after some species names refers to the phylogenetic position of chloroplast genes for duplicated taxa; RIBOSOME after some species names refers to the phylogenetic position of nuclear-ribosomal genes for duplicated taxa (for details, see Methods).

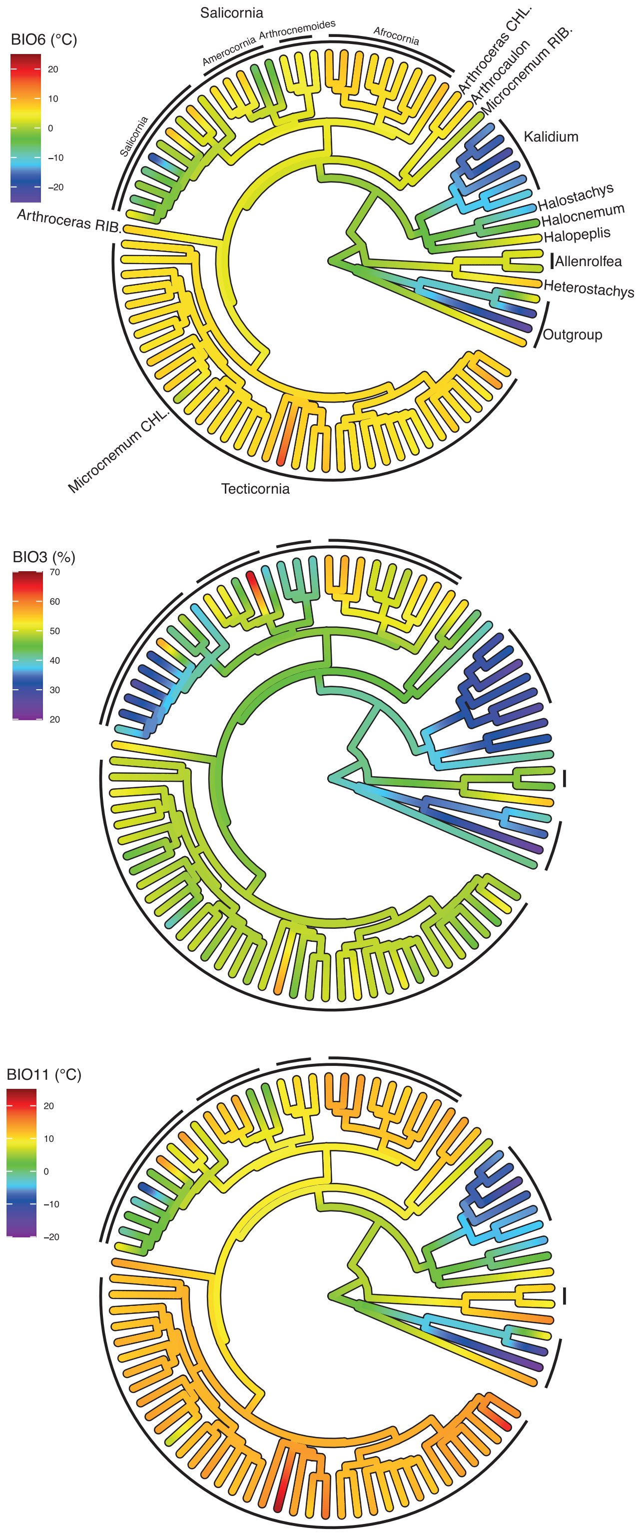

Figure 3. Ancestral state reconstruction of BioClim variables based on species means, including all species of Salicornieae (Salicornioideae, Amaranthaceae) with >10 occurrence records available in GBIF. Top: BIO6 = minimum temperature of the coldest month (°C). Middle: BIO3 = isothermality, i.e. annual temperature variation (among seasons) relative to diurnal temperature variation (between day and night). BIO3 is measured in percent, with a value of 100 indicating a diurnal temperature range equal to the annual range and anything below indicating lower diurnal than seasonal temperature variability. Bottom: BIO11 = mean temperature of the coldest quarter (°C). Black bars denote genera in Salicornieae plus subgenera of Salicornia and are shown instead of tip labels/species names (c.f. Figs. 1 and 4). CHL = chloroplast, and refers to the phylogenetic position of plastid loci for duplicated taxa; RIB = ribosome, and refers to the phylogenetic position of nuclear-ribosomal genes for duplicated taxa (for details, see Methods).

Sensitivity of the results to variation in frost tolerance coding

Recoding frost tolerance using BIO6 minima (95% CI lower bound) yields similar results for the corHMM and BayesTraits analyses as for the BIO6 means. The two-rate hidden rates model is still the best fitting (log likelihood (LL) = -43.6, ΔAICc > 5, Supporting Information, Table S9), as is the dependent model of correlated evolution (ML: $\chi^2 = 17.0$, d.f. = 4, $P < 0.01$. MCMC: logBF = 9.87). However, in both analyses scoring frost tolerance based on species minima yields models with lower likelihoods (−34.9 vs. −43.6 for corHMM and −67.0 vs. −72.7 for BayesTraits). The two datasets are not independent and differ only in how *c.* 10% of the data are scored; thus, the lower likelihoods could indicate that those models are suboptimal compared to the models based on species means. Furthermore, in the corHMM analysis, the uncertainty in the estimated root state of Salicornieae increased, the probability of the root being frost sensitive going from 0.75 to 0.54. The differences among the transition rate categories have also decreased (Table 2), and the fast rate has become more likely on many branches, notably including for the frost-tolerant *Salicornia* and *Kalidium* (Supporting Information, Fig. S10). For the BayesTraits analysis, the main inferred differences in probabilities of gains and losses remained the same (Supporting Information, Table S8). Overall, coding frost tolerance using species minima suggests the same overall scenario, but it is less precisely reconstructed.

DISCUSSION

An improved species-level phylogenetic hypothesis for Salicornieae

In this study we have increased species-level sampling in Salicornieae from 65–70% (Ball *et al.* 2017, Piirainen *et al.* 2017) to 85–90%, mostly by adding representatives of the species-rich genus *Tecticornia*. Sampling gaps still occur in *Allenrolfea*, *Arthrocaulon*, and the two most species-rich genera, *Salicornia* and *Tecticornia*. Our reconstructed phylogenetic tree confirms the results of previous studies, showing that the genera are mostly monophyletic, although *Tecticornia* is unresolved (Fig. 1). We resolve the deep polytomy found by Piirainen *et al.* (2017) and confirm the monophyly of the three main clades, finding that the American *Allenrolfea-Heterostachys* clade is sister to the rest of the tribe. The resolution of the deep divergences thus corroborates findings by Kapralov *et al.* (2006) and Morales-Briones *et al.* (2021), the latter based on multiple loci from transcriptomic data.

Resolution in the smaller clades is also improved here, although the positions of some clades and taxa, specifically *Arthroceras*, *Microcnemum* and *Kalidium schrenkianum*, remain uncertain. On the basis of morphology (Shepherd *et al.* 2005, Kadereit *et al.* 2006, Sukhorukov and Nilova 2016, Piirainen *et al.* 2017), the ribosome-supported position of *Microcnemum*

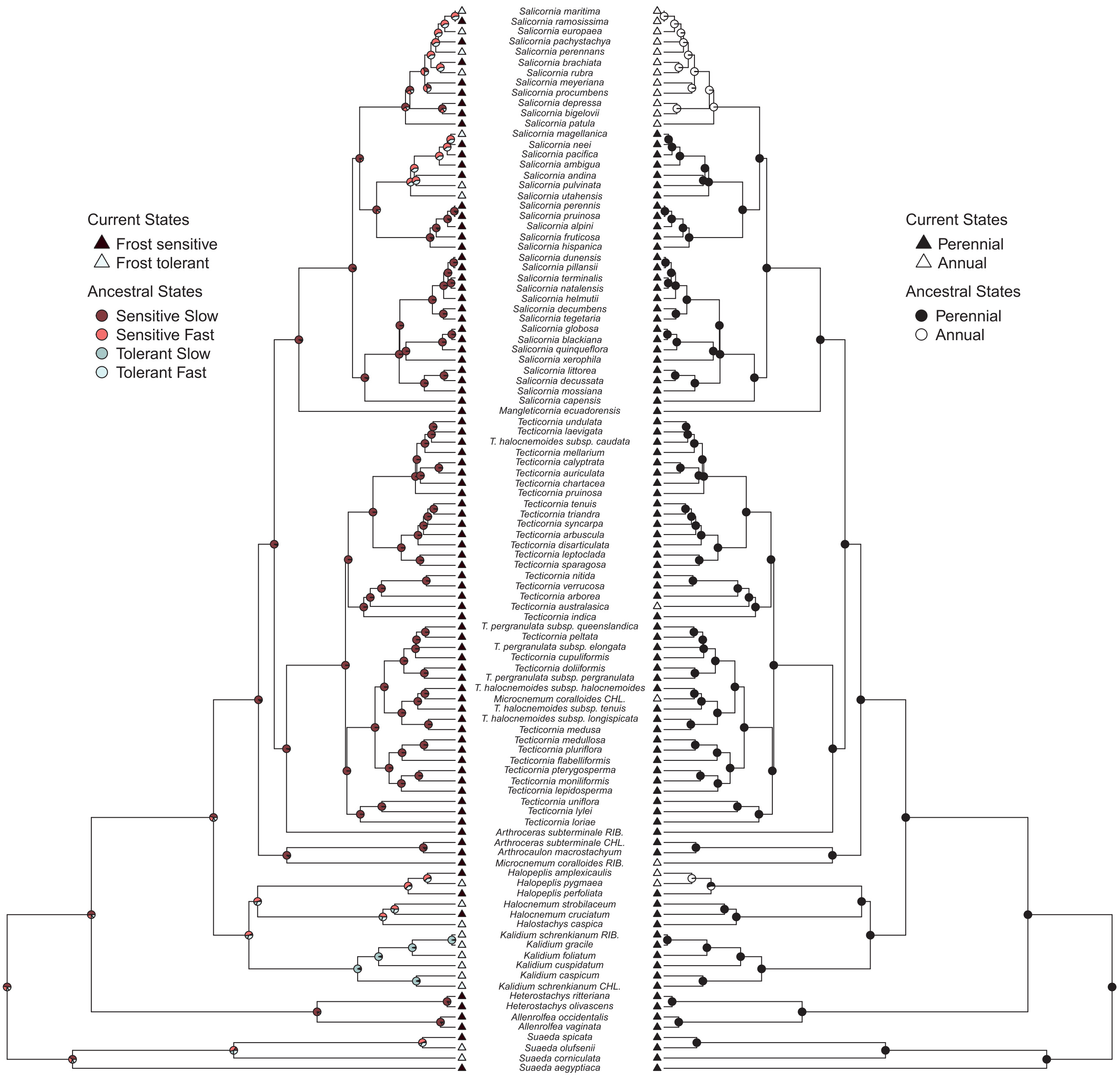

Figure 4. Ancestral state reconstruction of frost tolerance and life history in Salicornieae (Salicornioideae, Amaranthaceae). Left: frost tolerance. Right: life history. Pie charts indicate the probability of each state at each node, inferred as the frequency of each state sampled over 100 random restarts, according to the best-fitting hidden rates model (see Supporting Information, Table S9). Triangles at the tips show the binary state coding for frost tolerance and life history. Frost tolerance was scored as the average minimum temperature of the coldest month (BIO6) being <0°C. Tip labels: CHL = plastid, and refers to the phylogenetic position of plast loci for duplicated taxa; RIB = ribosome, and refers to the phylogenetic position of nuclear-ribosomal loci for duplicated taxa (for details, see Methods).

as sister to *Arthrocaulon* and a distant relationship between *Arthroceras* and *Arthrocaulon* seems more likely, but further study is needed to confirm this. We note that none of these taxa is particularly cold- or frost tolerant and this topological uncertainty is unlikely to influence downstream analyses. Similarly, the conflicting position of the frost-tolerant *Kalidium schrenkianum* will not influence results of those analyses. Finally, the polyphyletic nature of *Allenrolfea vaginata* remains; future phylogenetic research should therefore focus on resolving *Allenrolfea*, including the as-yet unsampled *A. patagonica*, and on *Tecticornia* and *Salicornia*.

Tecticornia, which was monophyletic but poorly sampled by Piirainen *et al.* (2017), stands out as being the only unsupported genus in the combined analysis. Previous studies have noted the limited genetic differentiation of morphologically distinct taxa in the clade, suggesting hybridization and rapid diversification as causes (Shepherd *et al.* 2005). We note that *Tecticornia*, being largely endemic to lowland regions of Australia (Fig. 2), does

Table 2. Transition rate matrices from the hidden rates analyses, estimated under (a,b) the two-rate category model for frost tolerance coded using species means (a) and species minima (b); and the one-rate category model for life history (c). Evolutionary transitions are expressed as relative rates.

(a) Frost tolerance with BIO6 species means

	Sensitive, Fast	Tolerant, Fast	Sensitive, Slow	Tolerant, Slow
Sensitive, Fast	NA	70	0.042	NA
Tolerant, fast	100	NA	NA	0.042
Sensitive, slow	0.020	NA	NA	10^{-9}
Tolerant, slow	NA	0.020	0.0012	NA

(b) Frost tolerance with BIO6 species minima

	Sensitive, Fast	Tolerant, Fast	Sensitive, Slow	Tolerant, Slow
Sensitive, fast	NA	1.8	0.040	NA
Tolerant, fast	1.3	NA	NA	0.040
Sensitive, slow	0.027	NA	NA	10^{-9}
Tolerant, slow	NA	0.027	0.013	NA

(c) Life history

	Perennial	Annual
Perennial	NA	0.011
Annual	0.036	NA

not harbour cold- or frost-tolerant species. The lack of phylogenetic resolution associated with *Tecticornia* is therefore unlikely to have influenced the results of our comparative analyses.

Salicornia is better resolved, with all but one of its four geographically and/or morphologically distinct subgenera being monophyletic [and the fourth being monophyletic barring one species, *S. capensis* (Moss) Piirainen & G.Kadereit]. The relationships of these subgenera are in concordance with Piirainen *et al.* (2017). Although polytomies persist in all subgenera, resolution is particularly poor in the original *Salicornia* (now subgenus *Salicornia*). This is perhaps not surprising given the history of the group: in a tribe of notoriously hard-to-delineate species it has been called the 'nightmare clade' (Kadereit *et al.* 2007, Kadereit *et al.* 2012b). The ancestrally reduced morphology of leaves and flowers means taxonomic definitions have often been based on size, habit or colour: traits generally considered to show high phenotypic plasticity (Vanderpoorten *et al.* 2011). Together with the tendency of the clade to develop habitually distinct ecotypes with little genetic divergence, this has given rise to many phylogenetically questionable taxon definitions (Teege *et al.* 2011, Vanderpoorten *et al.* 2011). Often, the geographical signal is stronger than the morphological signal in genetic analyses (Vanderpoorten *et al.* 2011), and many morphologically similar populations are phylogenetically distant (Kadereit *et al.* 2012a, Slenzka *et al.* 2013). Finally, inbreeding (Kadereit *et al.* 2007, Teege *et al.* 2011, Vanderpoorten *et al.* 2011) and hybridization (Murakeözy *et al.* 2007, Kaligarič *et al.* 2008) add further confusing signals. These factors are probably reflected in the short branch lengths, indicating limited genetic diversity, in most of *Salicornia* and subgenus *Salicornia* in particular (Fig. 1).

Evolution of cold and frost tolerance in Salicornieae

The ancestral state reconstructions estimate that the most recent common ancestor of Salicornieae occurred in an area with minimum temperatures between −8.7 and +6.8°C (mean estimate −0.94°C; Fig. 3), whereas the hidden rates model finds a probability of 0.25 that the ancestral area experienced frost (Fig. 4). Both analyses thus indicate considerable uncertainty regarding whether the tribe was ancestrally adapted to freezing conditions, although they were most probably cold enough to cause chilling stress (Nievola *et al.* 2017). Taken at face value, a minimum temperature distributed around 0 (with a slightly higher probability of there being no frost), coupled with an isothermality (BIO3) between 30 and 50%, would suggest ancestral conditions of the tribe akin to Mediterranean or subtropical climates of today (Peel *et al.* 2007, Fick and Hijmans 2017). This is consistent with an origin of Salicornieae in the Oligocene (as found here, Supporting Information, Fig. S7; and elsewhere, Piirainen *et al.* 2017). The onset of the Oligocene saw a significant drop in temperature (Zachos *et al.* 2001), and most of the epoch saw markedly lower temperatures than the Eocene before it, when the tropics stretched as far north as Germany (Grein *et al.* 2011). However, temperatures were still higher than today, meaning that the Mediterranean Basin and western Asia, where Salicornieae most probably originated (Piirainen *et al.* 2017), would have experienced a Mediterranean to subtropical climate. Thus, although it is unclear exactly how susceptible the last common ancestor of Salicornieae was to cold and frost, it was probably tolerant of chilling but not subjected to many frost events.

The ancestral state reconstruction of cold tolerance further suggests that most lineages have retained their ancestral climatic niches or dispersed into warmer areas, and that only *c.* 10% of species experience temperatures markedly colder than reconstructed at the root (Fig. 3, Supporting Information, S1). This is in line with the hypothesis that most clades retain their ancestral niches (Crisp *et al.* 2009, Wiens *et al.* 2010), and that overcoming the barriers imposed by cold climates is a significant challenge (Latham and Ricklefs 1993). Today, cold tolerance appears to peak in certain lineages of *Salicornia* and in the *Kalidium-Halocnemum-Halopeplis-Halostachys* clade. These clades are also the ones most likely to be subjected to frost based on their geographical distributions (Fig. 2). They all experience greater seasonal variation in temperature (BIO3, Fig. 3) than in the ancestral area of Salicornieae, whereas *Kalidium* in particular experiences low temperatures (BIO6 and BIO11, Figs 2, 3). Internal nodes that indicate frost tolerance with certainty (PP > 0.95) are confined to *Kalidium*, although the probability of ancestral frost tolerance is also higher in its close relatives and for some nodes of *Salicornia*. Taken together, these results indicate at least two independent transitions into cold climates.

At the core of the corHMM method is the theory of 'hidden rates', qualities that influence trait evolution by increasing or decreasing the rate of change (Beaulieu *et al.* 2013). These qualities can be interpreted as 'precursor traits' (Marazzi *et al.* 2012), traits that facilitate evolution of other traits. Latham and Ricklefs (1993) suggested that it may have been such precursors for frost tolerance that allowed certain subtropical clades to adapt to temperate conditions when the global climate cooled after the end of the Eocene. In this study, the hidden rates analysis finds that frost tolerance evolution is best described by two transition rates. The slower rate is somewhat more probable for the deeper nodes and it dominates most younger nodes of the tree (Fig. 4). Most of these lineages are inferred to be frost sensitive. The faster rate is most prevalent in *Salicornia* subgenera *Amerocornia* and *Salicornia*, but also increases in probability in *Halocnemum*, *Halopeplis* and *Halostachys*. All these clades harbour present-day frost-tolerant species. In contrast, the fast transition rate is improbable in *Kalidium* (probability < 0.09), despite *Kalidium* being the only clade in Salicornieae that is exclusively frost tolerant. This result can be interpreted as being indicative of there being different frost tolerance mechanisms in Salicornieae, one in *Kalidium*, indicated by the frost-tolerant-slow state, and the other in its sister clade plus two subgenera of *Salicornia*, indicated by the frost-tolerant-fast state. These results therefore suggest as many as four independent gains of frost tolerance in the tribe, two in *Salicornia*, one in *Halocnemum-Halopeplis-Halostachys* and one in *Kalidium.* Further, our results point to these independent gains being facilitated by one or more precursor traits: the fast transition rate occurs exclusively in lineages where frost tolerance has evolved and appears more labile. This suggests frost-tolerant lineages in the fast rate category can be interpreted as having arisen by modification of evolutionary precursor traits, or 'enablers', as has been inferred for repeated evolution of C_4 photosynthesis and the annual life history strategy in grasses (Christin *et al.* 2013, Lindberg *et al.* 2020, Hjertaas *et al.* 2022). The ancestral state reconstruction is not certain enough at deeper nodes to determine when, exactly, such precursor(s) might have evolved, or whether it might have been ancestral in the tribe.

If Salicornieae originated in the Oligocene, the early diversification and divergence of the three main clades occurred in the Oligocene and early Miocene. Temperatures rose significantly at the end of the Oligocene and then stayed comparatively stable until the Mid-Miocene Climatic Optimum 15 Myr ago, when they started to decrease (Zachos *et al.* 2001, Mudelsee *et al.* 2014). This caused the climate to become drier, probably resulting in the expansion of halophytic environments and possibly enabling expansion of Salicornieae (Piirainen *et al.* 2017). As cooling progressed, the ancestors of present-day cold-tolerant clades most probably possessed the right traits (which acted as precursors or enablers) to be able to adapt *in situ* as the climate cooled around them, while less hardy or adaptable lineages went extinct (see also Nürk *et al.* 2015, Schubert *et al.* 2020). Indeed, we find a phylogenetic pattern of long branches leading to relatively species-poor clades with shorter internal branches in all but one of the Northern Hemisphere clades. Such a pattern is typically interpreted as the result of extinction (Crisp and Cook 2009, Spriggs *et al.* 2015, Humphreys *et al.* 2016), an interpretation that has also been applied to Salicornieae (Kadereit *et al.* 2006, 2007). In the especially cold-tolerant *Kalidium*, our findings support a scenario where all sensitive taxa became extinct, explaining the absence of frost tolerance in the fast rate category in this clade.

What, then, enabled certain lineages of Salicornieae, but not all, to adapt to cold? The ancestrally high abiotic stress tolerance of the tribe (Kadereit *et al.* 2012a) may have been important, as drought, salt and frost impose many similar physiological challenges (Preston and Sandve 2013, Folk *et al.* 2020, Schat *et al.*, **unpubl. data;** Stolsmo *et al.*, **unpubl. data**). Sakai and Larcher (1987) suggested that the osmotic adjustments necessary to survive drought, adjustments that are also critical in dealing with high salt levels (Weber 2009, Meng *et al.* 2018), may have enabled evolution of cold tolerance. Responses to cold stress may have been co-opted from ancestral responses to general cellular stress (indicated in Pooideae; Schubert *et al.* 2019b), and high intracellular salinity caused by halophytic environments may have assisted osmotic adjustment to frost, as seen in *Atriplex* L. (Martínez *et al.* 2005, Walker *et al.* 2008, Aouissat *et al.* 2009, Walker and Lutts 2014). Another interesting trait is seed dormancy cycling, seen in both the annual *Salicornia europaea* L. (Carter and Ungar 2003) and perennial *Kalidium gracile* Fenzl (Cao *et al.* 2014). In Mongolia, *K. gracile* seeds primarily germinate in the early growing season when temperature and soil water content are high and soil salinity low (Cao *et al.* 2014), thus linking life history traits to cold, drought and salt tolerances. Dormancy cycling and seed dimorphism also enable establishment of persistent seed banks (Carter and Ungar 2003, Cao *et al.* 2014), which could allow even perennial species to re-establish populations after particularly harsh winters. The ancestral ability to withstand various abiotic stresses may thus have facilitated the evolution of life history traits and physiological responses to cold, allowing hardy species to adapt as the climate cooled.

Adaptation in growth form may be an explanation for cold adaptation in perennial *Salicornia*, where cold-tolerant species may escape frost or snow by forming subterranean branches (mat-forming species) or adopting a prostrate or pulvinate habit (Kadereit *et al.* 2006, Alonso and Crespo 2008), and where populations experiencing colder conditions appear to grow closer to the ground (Steffen *et al.* 2015). Such mat-forming habits have arisen multiple times within perennial lineages of *Salicornia* (Steffen *et al.* 2015). For example, in the mat-forming *S. magellanica* Phil. and the creeping *S. pacifica* Standl., frost might strongly affect aerial parts of the plants and select for decumbent growth forms. In other mat-forming or prostrate species of the genus, regular flooding with high drag from tidal movement seems to have selected for the parallel evolution of similar habits, e.g. in the South African *S. tegetaria* (Steffen, Simone, Mucina & G.Kadereit) Piirainen & G.Kadereit and the Australian *S. quinqueflora* Bunge ex Ung.-Sternb. (Steffen *et al.* 2015).

Further comparative or experimental studies are needed to properly assess these ideas and to examine cold-tolerant taxa for other promising traits (e.g. physiological ones). However, such studies might reveal that the phenotypic differences between cold-tolerant and cold-sensitive clades are minimal, and that certain taxa occur in cold areas today because they were able to adapt *in situ* as the climate cooled around them (c.f. Nürk *et al.* 2015, Schubert *et al.* 2019a, 2020); in other words because they were exposed to the extrinsic pressure to repurpose ancestral stress tolerance traits in a certain way.

No evolutionary relationship between frost tolerance and an annual life history

The annual life form occurs in two species of *Halopeplis*, one species of *Tecticornia*, in *Microcnemum*, and in *Salicornia* subgenus *Salicornia*. It coincides with frost tolerance only in *Halopeplis pygmaea* (Pall.) Bunge ex Ung.-Sternb. and in *Salicornia* subgenus *Salicornia* (Fig. 4). Despite this, the BayesTraits analysis rejects the null hypothesis that annuals and frost tolerance have evolved independently. However, our simulations reveal that this is due to a type I error, a problem that is common for a range of macroevolutionary methods (Supporting Information, Fig. S9; Maddison and Fitzjohn 2015, Rabosky and Goldberg 2015, Humphreys *et al.* 2016, Moore *et al.* 2016). Instead, across Salicornieae as a whole, frost tolerance evolution occurs independently of life history evolution. For the clades that contain frost-tolerant annuals, the separate ancestral state reconstructions suggest that annuality evolved first (Fig. 4), suggesting that the annual life history strategy could have been a prerequisite for, rather than adaptation to, a freezing climate.

Compared to perennials, annuals generally have a fast growth rate and short juvenile period (Verboom *et al.* 2004, Bergonzi and Albani 2011, Hjertaas *et al.* 2022). This means annuality has been considered adaptive in areas with short growing seasons (Friedman 2020). Annual Salicornieae may persist in cold areas because they are able to complete their life cycles in a short time, surviving the worst of the winter as seeds and using dormancy cycling to germinate at an opportune moment (Carter and Ungar 2003). These features may originally have allowed colonization of areas with seasonal flooding or varying salt and drought levels (Kadereit *et al.* 2007, Cao *et al.* 2014) and may thus be interpreted as facilitating adaptation to seasonally cold and/or freezing conditions. Although annuality is unlikely to offer a complete escape strategy, as annuals in cold areas still have to contend with both occasional frosts during the growing season and establish seedlings in adverse conditions (Körner 2003), it may serve as a partial escape allowing quicker adaptation to cold climates. Annual clades would thus represent an evolutionary route into cold areas, separate from the one(s) taken by frost-tolerant perennials. Future research may determine whether annuals and perennials also use different physiological and molecular mechanisms to overcome the stresses posed by cold and freezing temperatures.

Limitations and outlook

Analysing bioclimatic data can be challenging, particularly when the raw, quantitative variables are to be coded as binary traits (e.g. frost tolerant/frost sensitive). Results can be influenced by the choice of critical limit (e.g. the temperature at which a species is considered tolerant; Schubert *et al.* 2019a) or by the use of species means, minima, or other values (e.g. in this study the probably frost-tolerant *S. procumbens* and *S. andina* do not have BIO6 means below 0°C). The best way to ensure accurate coding is manual inspection; but, this becomes unrealistic as datasets grow to include hundreds or even thousands of species. Therefore, it is encouraging to see that recoding the data here, using species minima instead of means, produced the same overall results (Supporting Information, Fig. S10, Tables 2, S8 and S9). Schat *et al.* **(unpubl. data)** came to a similar conclusion when experimenting with different critical limits in their analysis of frost tolerance in grasses. Notably, both their analysis and ours found that, although qualitative results (e.g. model selection) were robust to alternative treatments of the data, quantitative results (e.g. parameter estimates) were more variable. This means biological conclusions might for the most part be robust to alternative treatments of the data, but the nuances can still be affected (see also Edwards *et al.* 2015), emphasizing the importance of testing the robustness of conclusions to alternative treatments of the data.

Humphreys and Linder (2013) showed that realized niches estimated from geographical occurrence records can be a good proxy for fundamental cold tolerance. However, they stress that this finding may not be general. Occurrence data are difficult to process given their varying accuracy (Zizka *et al.* 2020b) and run the risk of underestimating the occupied niches of species due to lack of geographical observations. Taxa observed in freezing areas can thus generally be said to experience frost, but the reverse is not necessarily true. In addition, if species distributions are strongly structured along microclimatic gradients (e.g. Greiser *et al.* 2020), the coarse-grid occurrence data used here may both over- and under-estimate frost tolerance. Further, the temperature data used here are air temperatures, which may differ from the conditions felt at ground level, the level most relevant for small-stature plants (e.g. Still *et al.* 2014). Finally, the multitude of factors that define realized niches may mean realized niches do not accurately represent differences in inherent cold tolerance among clades. However, this is unlikely to bias our conclusions, as differences related to inferred versus inherent physiological stress responses are more likely to influence species-level differences than the broader patterns that, regardless of the qualities causing them, are clearly evident.

The limitations outlined here all open new, intriguing avenues for future research. The most obvious is tests of fundamental cold tolerance limits. Common garden experiments have been performed for Salicornieae (Teege *et al.* 2011), as have reciprocal transplants (Davy and Smith 1985), but never to study cold tolerance. Performance in controlled climates and in further field experiments would allow comparison of the cold tolerance of species that occur in freezing areas and species that do not, providing valuable insights into whether relative cold tolerance levels match expectations based on our findings here. Moreover, future studies with increased sampling of the sister clades of Salicornieae might improve inference of the ancestral cold tolerance in the broader clade. In this study, *Suaeda spicata*, one of the few outgroups from a warmer habitat with available data, is still coded as frost tolerant when using species minima (Supporting Information, Fig. S10). Although this does not influence the conclusions of this study (as indicated by preliminary analyses where the taxon was removed), it nevertheless indicates a sampling skew towards colder habitats in the outgroup, which could be circumvented by better sampling of warm climate taxa in the future. Furthermore, (eco) physiological studies will be important to determine whether there are different cold and frost responses (adaptations) in different lineages, as has been indicated in grasses (Schubert *et al.* 2019b, 2020). Especially interesting is the comparison between *Kalidium* and *Salicornia* and, within *Salicornia*, comparative physiology of species in different subgenera and with different life history strategies. Determining whether cold adaptations vary significantly among lineages will serve to support or refute the findings here, of multiple independent gains of cold tolerance across the tribe.

CONCLUSIONS

We provide an updated and broadened phylogenetic tree for Salicornieae, which confirms previous studies and resolves some previously uncertain relationships. We show that the species of Salicornieae occupying the coldest climates occur primarily in Eurasia and North America and in two phylogenetically disparate clades. Ancestral state reconstructions indicate that Salicornieae was possibly ancestrally adapted to mild chilling stress but that adaptations to severe cold and frost most likely evolved later, at least twice and perhaps as many as four times independently, each time most probably by *in situ* adaptation to changing climates as opposed to colonization of freezing regions *de novo*. A probable enabler was therefore being present in the right place at the right time, along with the high ancestral abiotic stress tolerance of the clade. These findings add to the growing picture of tropical-to-temperate transitions having occurred more frequently across flowering plants than widely assumed.

SUPPLEMENTARY DATA

Supplementary data is available at *Botanical Journal of the Linnean Society* online.

Table S1. Taxa and GenBank accessions included in the analysis.

Table S2. GenBank accession numbers and voucher information for DNA sequences generated in this study.

Table S3. PCR primers used in this study.

Table S4. PCR programmes used in this study.

Table S5. Alignment summary statistics.

Table S6. Nucleotide substitution models used in the phylogenetic analyses.

Table S7. Correlation tests for pairwise comparisons of mean, minimum, and maximum BioClim values.

Table S8. Parameter definitions and transition rate estimates for Pagel's model of correlated evolution.

Figure S1. RAxML gene tree for the External Transcribed Spacer (ETS).

Figure S2. RAxML gene tree for the Internal Transcribed Spacer (ITS).

Figure S3. RAxML gene tree for the *atpB-rbcL* spacer.

Figure S4. RAxML gene tree for the *matK-trnK* intron.

Figure S5. Concatenated nrDNA (ETS + ITS) tree.

Figure S6. Concatenated cpDNA (*atpB-rbcL* + *matK-trnK*) tree.

Figure S7. Dated tree estimated in Beast using all concatenated data (nr- and cpDNA).

Figure S8. BayesTraits model convergence plots.

Figure S9. Frequency plot of difference in fit between independent and dependent models of evolution for simulated data.

Figure S10. Ancestral state reconstruction under the hidden rates model (corHMM) and frost tolerance scored using species minima (sensitivity analysis).

Data S1. Taxon list and BioClim means for the cold tolerance analysis (separate .csv file, deposited in Dryad; doi.org/10.5061/dryad.573n5tb9v; Humphreys *et al.* 2022).

Data S2. Taxon list and binary coding for frost tolerance and life history (separate .csv file, deposited in Dryad; doi.org/10.5061/dryad.573n5tb9v; Humphreys *et al.* 2022).

Table S9. Hidden rates model (corHMM) diagnostics.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY

The DNA sequence alignments (full and reduced), resulting consensus trees (MrBayes for the full alignment; dated Beast tree for the reduced alignment) and the climate and life history data (BioClim means and binary coding for life history and frost tolerance; Supplementary Data S1 and S2) are available via Dryad (doi.org/10.5061/dryad.573n5tb9v; Humphreys *et al.* 2022).

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