On the hybrid origin of the C₂ *Salsola divaricata* agg. (Amaranthaceae) from C₃ and C₄ parental lineages

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Abstract

- C₂ photosynthesis is characterized by recapturing photorespiratory CO₂ by RuBisCO in Kranz-like cells and is therefore physiologically intermediate between C₃ and C₄ photosynthesis. C₂ is either interpreted as an evolutionary precursor of C₄ or as the result of hybridization between a C₃ and C₄ lineage.
- We compared the expression of photosynthetic traits among populations of the *Salsola divaricata* agg. (C₂) from humid subtropical to arid habitats on the coasts of the Canary Islands and Morocco, and subjected them to salt and drought treatments. We screened for enhanced C₄-like expression of traits related to habitat or treatment. We estimated species trees with a transcriptome dataset of Salsoleae and explored patterns of gene tree discordance. With phylogenetic networks and hybridization analyses we tested for hybrid origin of the *Salsola divaricata* agg.
- We observed independent variation of photosynthetic traits within and among populations and no clear evidence for selection towards C₄-like trait expression in more stressful habitats or treatments. We found reticulation and gene tree incongruencies in the Salsoleae supporting a putative hybrid origin of the *Salsola divaricata* agg.
- C₂ photosynthesis in the *Salsola divaricata* agg. combines traits inherited from its C₃ and C₄ parental lineages and seems well adapted to a wide climatic amplitude.

Keywords: C₂ photosynthesis, carbon isotope values, CO₂ compensation point, Chenopodiaceae, phosphoenolpyruvate carboxylase, *Salsola divaricata* agg., phylogenomics, hybridization events.

Introduction

In current models of C₄ evolution, the C₃-C₄ intermediate phenotypes (including C₂ plants) are interpreted as a transitional, evolutionary link between the ancestral C₃ photosynthesis pathway and the derived C₄ pathway and showcase the complexity of the numerous structural, genetic and functional changes necessary to establish a functioning C₄ pathway (Bräutigam & Gowik, 2016; Schlüter & Weber, 2016). There are about 50 known species with intermediate photosynthetic traits, some of which do not share a common ancestor with one of the more than 60 independent C₄ lineages (Sage *et al.*, 2011), and some of which represent lineages up to 20 or even 30 million years in their crown age (Sage *et al.*, 2018). The fact that we are able to observe these intermediate phenotypes in nature repeatedly implies that they are evolutionary stable (Lundgren, 2020). Their phenotypic diversity has been classified into four photosynthetic categories of C₃-C₄ intermediacy: proto-Kranz, C₂ type I, C₂ type II and C₄-like (Sage *et al.*, 2014). In particular, C₂ photosynthesis has been interpreted as a crucial stepping stone towards C₄ photosynthesis. In C₂ species the photorespiratory enzyme glycine decarboxylase (GDC) is restricted to the bundle sheath or Kranz-like cells and mitochondria are absent or distinctly reduced in the mesophyll cells. This induces a photorespiratory glycine shuttle to the bundle sheath cells where the glycine is then processed by GDC and the photorespiratory CO₂ is recaptured by RuBisCO (Schulze *et al.*, 2016).

Bräutigam & Gowik (2016) propose that once the photorespiratory CO₂ pump is active, establishing the C₄ cycle is inevitable. According to this model, a strong selective pressure towards an increase of phosphoenolpyruvate carboxylase (PEPC) activity in C₂ species should be expected under carbon deficient conditions (Bräutigam & Gowik, 2016).

An alternative to the evolution of C₃ to intermediates to C₄ is the proposed occurrence of intermediates through hybridization (Monson *et al.*, 1984). Experimental hybrids of C₃ and C₄ species (e.g., Oakley *et al.*, 2014) reveal similar phenotypes as naturally occurring C₃-C₄ intermediate species (Brown & Bouton, 1993). Furthermore, conflicting topologies in molecular phylogenetic studies which include C₃-C₄ intermediate species indicate possible hybridization events in the evolutionary history of these lineages (reviewed in Kadereit *et al.*, 2017). To investigate the possibility of a hybrid origin of a C₂ lineage, detailed phylogenomic studies are needed; however, such a study has not been conducted so far.

We conducted a study of a C₂ species at the population level and looked at C₄-adaptive traits to provide empirical evidence for one of the two alternative hypotheses. In the case of C₃-C₄ intermediates being evolutionary stepping stones and according to the model proposed by Bräutigam & Gowik (2016), we would expect a pattern of populations showing shifts towards more C₄-like traits under stressful conditions. In the case of C₃-C₄ intermediates resulting from hybridization, we would expect independent segregation of C₄-traits as has been found in several hybridization experiments (Björkman *et al.*, 1969; Holaday *et al.*, 1985; Cameron *et al.*, 1989; Brown *et al.*, 1993; Brown & Bouton, 1993; Oakley *et al.*, 2014).

The C₂ species we chose for our study is *Salsola divaricata* Moq. (Amaranthaceae, subfamily Salicornioideae, tribe Salsoleae; Schüssler *et al.*, 2017; Morales-Briones *et al.*, 2021) and its close relatives *S. verticillata* Schousb., *S. gymnomaschala* Maire and *S. deschaseauxiana* Litard. & Maire (hereafter called the *S. divaricata* agg.). The distribution area of the *Salsola divaricata* agg. on the Canary Islands and the coasts of Western Morocco, spans a considerable climatic gradient with an arid climate in the East and a mesic mediterranean climate in the West (García-Herrera *et al.*, 2001).

Taxa of the *Salsola divaricata* agg. are salt tolerant, perennial shrubs with terete succulent leaves and morphologically difficult to distinguish (Padrón Mederos, 2012). They all have a Kranz-like salsoloid leaf anatomy (Voznesenskaya *et al.*, 2013; Schüssler *et al.*, 2017). *Salsola divaricata* was categorized as a C₂ type I which is characterized by the photorespiratory CO₂ pump through GDC being confined to the bundle sheath cells while having C₃-like activity of C₄ enzymes (e.g. PEPC and NADP-ME). Their CO₂ compensation points around 30 μmol mol⁻¹ are intermediate to C₃ and C₄ species (Schüssler *et al.*, 2017). The *Salsola divaricata* agg. belongs to Salsoleae, a tribe rich in C₄ species, but also with several C₃ species and a number of C₃-C₄ species. The *Salsola divaricata* agg. forms a well-supported but unresolved subclade within a clade of C₃-C₄ intermediate and C₃ species (Schüssler *et al.*, 2017).

The aim of this study was to find empirical evidence either for the model of C₄ evolution through C₂ as an intermediate pathway or for C₂ being the result of hybridization of a C₃ and C₄ parental lineage. We studied C₄ adaptive traits at the population level in the C₂ lineage *S. divaricata* agg. and searched for locally adapted more C₄-like phenotypes. We also generated 991 gene trees from a transcriptome data set comprising C₃, C₄ and C₂ species of the Salsoleae and explored incongruences to determine if a hybridization event is plausible in the lineage of the *S. divaricata* agg.

Fig. 1: Map of the collection locations of seeds and vouchers for the *S. divaricata* agg. (see also **Supporting Information Table S1** for voucher information). In total we analyzed 26 populations of the *S. divaricata* agg. on the Canary Islands and Morocco. Species name abbreviation and population number: Sd = *Salsola divaricata*, Sg = *S. gymnomaschala*, Sv = *S. verticillata*, Sx = *S. deschaseauxiana*; Pxxx = population number. Note: Morphological studies on the species complex (Brullo, 1982; Fennane and Ibn Tattou, 1998) consider *S. verticillata* and *S. deschaseauxiana* as conspecific, while *S. gymnomaschala* seems more similar to *S. divaricata* s.s.

Materials and Methods

Plant Material

Seeds and vouchers of 26 populations of the *S. divaricata* agg. were collected in 2013 and 2014 in the Canary Islands and Morocco (**Supporting Information Table S1**; **Fig. 1**) and grown in a greenhouse at the Botanical Garden Mainz with multiple individuals per population. Plants were grown in custom mixed soil. Day/night cycles of 14h/10h were artificially maintained with natural light and supplementary light with a total light intensity of 200–400 $\mu\text{mol m}^{-2} \text{s}^{-1}$. The minimum temperature at night was 18 °C and daytime temperatures ranged between 25 to 35 °C in summer and 20 to 25 °C in winter. All plants were watered once a week in the winter and twice a week in the summer. The individuals included in the salt treatment were watered with 2% saltwater (20 g NaCl per liter; 34.3 mM) every two weeks from June 2015 until 2021. Individuals in the dry treatment were watered every two weeks from June 2015 to March 2017. The analyses of the photosynthesis traits began in 2016. **Supporting Information**

Table S1 lists the populations included in this study as well as the collection and voucher information.

Carbon isotope measurements

C₃ plants discriminate against fixing atmospheric ¹³CO₂ by RuBisCO while C₄ plants do not discriminate via PEP carboxylase resulting in different isotope composition in plant biomass. Therefore, carbon isotope values (δ¹³C) can be used to detect C₄ cycle activity (Sage, 2016). We determined δ¹³C values between and within six populations of *S. divaricata* with six individuals per treatment (control, dry and salt). We also measured 30 samples collected from wild mother plants of 24 populations and compared them with the greenhouse cultivated offspring (165 individuals in total, all populations, including 36 in dry and 36 in salt treatment). To determine carbon isotope composition, a standard procedure with PDB (Pee Dee Belemnite) limestone as the carbon isotope standard was used (Bender *et al.*, 1973). The leaf samples were dried in silica gel, and then 1–2 mg were placed in a tin capsule and combusted in an EuroVector elemental analyser (EuroVector, Italy). After separating the resulting N₂ and CO₂ gases by gas chromatography, they were fed into the IsoPrime™ isotope ratio mass spectrometer (IRMS; GV Instruments Ltd. (Micromass Ltd.), United Kingdom) for determination of ¹³C/¹²C ratios (R). δ¹³C values were determined according to δ¹³C(‰)=1000(R_{sample}/R_{standard})⁻¹, where R is ¹³C/¹²C. The measurements were taken at Washington State University and at the Geology Department at the University of Mainz.

CO₂ compensation point measurements

The CO₂ compensation point is a trait which is often used to identify C₄ species due to their very low values (usually less than 4 μmol CO₂ mol⁻¹). We measured one to two individuals (1 to 3 technical replicates for each individual) of two to four populations per island of all species in the *S. divaricata* agg. (in total 53 individuals). Measurements were made on the unstressed group and four individuals in the dry and salt treatment. C₃ *S. webbia* (2x) and C₄ *S. oppositifolia* (3x) were measured for comparison. CO₂ compensation points were determined using the portable gas exchange measurement system GFS-3000 (Heinz Walz GmbH, Germany) with the standard measuring head 3010-S equipped with a standard leaf area cuvette and a LED-Array (for details see **Supporting Information Material & Methods S1**).

PEPC activity measurements

Phosphoenolpyruvate carboxylase (PEPC) plays an important role in the C₄ pathway as it fixes atmospheric CO₂ after its conversion to HCO₃⁻ by carbonic anhydrase into a four-carbon compound which gave this photosynthesis pathway its name (Reyna-Llorens & Hibberd, 2017). C₄ plants show high levels of PEPC activity compared to C₃ plants and an increased activity is seen as an important stage in evolving from an intermediate to a full C₄ photosynthesis pathway (Sage *et al.*, 2012). In total we sampled 14 populations of *S. divaricata* and six from the other three species (predominantly analyzing 5 to 7 individuals per population). Treatments were compared with 38 individuals in the control group, 23 in salt, and 17 in the dry treatment after quality check. PEPC activity measurements were conducted using the Tecan infinite M1000 (Tecan Trading AG, Männedorf, Switzerland) and a Greiner UV-Star 96-well plate (Sigma-Aldrich Chemie GmbH, Munich, Germany). For details of sample preparation see **Supporting Information Material & Methods S1**.

The assay of PEPC was initiated by adding leaf extract and 3.9 mM phosphoenolpyruvate (PEP). The chlorophyll content of the leaf extract was measured according to Wintermans and De Mots (1965) in 96% ethanol. PEPC activity (*a*) was calculated on the basis of the linear part of the progress curve as

$$a = SU[\mu\text{mol ml}^{-1} \text{ min}^{-1}]V_{\text{assay}}[\text{ml}]/(1000V_{\text{enzyme}}[\text{ml}]C_{\text{Chla+b}}[\text{mg ml}^{-1}]),$$

where SU is the substrate turnover, *V*_{assay} is the total assay volume, *V*_{enzyme} is the volume of the leaf extract and *C*_{Chla+b} is the concentration of chlorophyll in the leaf extract. Statistical analyses were conducted in R v3.1.2 (R Core Team, 2014).

Transcriptome processing and nuclear phylogenetic analyses

To evaluate the potential hybrid origin of the *S. divaricata* agg., we generated gene trees from a transcriptome dataset comprising C₃, C₄ and C₂ species of Salsoleae; in total 13 transcriptomes representing 12 species. Three samples of these were newly sequenced on an Illumina HiSeq2500 platform at the University of Minnesota Genomics Center (paired-end 125 bp). For RNA extraction protocol and library preparation refer to Morales-Briones *et al.* (2021). Additionally, we sampled two species of Camphorosmeae and one of Caroxyloneae, and *Beta vulgaris* L. subf. Betoideae - Amaranthaceae as outgroups. **Table 1** lists all samples with their photosynthesis type, ploidy if known and SRA accession number. We followed Morales-Briones *et al.* (2021) for raw read processing, transcriptome assembly, transcript clustering, and homolog tree inference. We then applied the ‘monophyletic outgroup’

(MO) approach of Yang & Smith (2014) to prune orthologs that have all 17 taxa present. We used concatenation and coalescent-based methods for phylogenetic reconstruction. For the concatenation approach, we prepared a supermatrix by keeping only ortholog alignments with at least 300 bp and all 17 taxa. We estimated a maximum likelihood (ML) tree with RAxML v 8.2.11 (Stamatakis, 2014) using a partition by gene scheme. Clade support was assessed with 100 rapid bootstrap (BS) replicates. To estimate a species tree that is statistically consistent with the multi-species coalescent (MSC), Individual gene trees were used to infer a species tree using ASTRAL-III v.5.6.3 (Zhang *et al.*, 2018) using local posterior probabilities (LPP; Sayyari & Mirarab, 2016) to assess clade support. To examine nuclear gene tree discordance, we first calculated the Internode Certainty All score (ICA; Salichos *et al.*, 2014). Also, we calculated the number of conflicting and concordant bipartitions on each node of the species trees. We calculated both the ICA scores and the number of conflicting and concordant bipartitions with Phyparts (Smith *et al.*, 2015). Additionally, to distinguish strong conflict from weakly supported branches, we evaluated tree conflict and branch support with Quartet Sampling (QS; Pease *et al.*, 2018) using 1,000 replicates (for details see **Supporting Information Material & Methods S1**).

Assessment of hybridization

In order to detect possible hybridization, first we inferred species networks under a maximum pseudo-likelihood (Yu & Nakhleh, 2015) approach using PhyloNet v.3.6.9. (Than *et al.*, 2008). To estimate the best number of hybridizations and test whether the species network fits our gene trees better than a strictly bifurcating tree, we performed model selection using the bias-corrected Akaike information criterion (Sugiura, 1978) and the Bayesian information criterion (Schwarz, 1978). We also used HyDe (Blischak *et al.*, 2018) to estimate the amount of admixture (γ) in putative hybrid lineages. We tested all triples combinations and significance was assessed with a Bonferroni correction (for details see **Supporting Information Material & Methods S1**).

Plastome assembly and phylogenetic analysis

To investigate phylogenetic signal from plastid sequences, *de novo* assemblies were carried out with the Fast-Plast v.1.2.6 pipeline (<https://github.com/mrmckain/Fast-Plast>) using the filtered organelle reads obtained from the transcriptome raw read processing. A ML tree was inferred with IQ-TREE v.1.6.1 (Nguyen *et al.*, 2015) using the automated model selection (Kalyaanamoorthy *et al.*, 2017) and 200 standard non-parametric BS replicates for branch

support (for details see **Supporting Information Material & Methods S1**). Additionally, we used QS with 1,000 replicates, to detect potential plastome conflict in the backbone as seen in other groups of Amaranthaceae s.l. (Morales-Briones *et al.*, 2021).

Analysis of photosynthetic gene trees

We inferred trees for 50 genes that are important in photosynthesis to determine if the genes in *Salsola divaricata* agg. show a tendency to group with either a C₃ or a C₄ species. We downloaded coding and protein sequences from *Arabidopsis thaliana* as baits from the TAIR website (<https://www.arabidopsis.org/tools/bulk/sequences/index.jsp>). These loci were determined based on Lauterbach *et al.* (2017a,b) and included two RuBisCO subunit genes, four genes coding for proteins of the glyoxylate cycle, 17 genes encoding photorespiratory proteins, and 25 genes coding for C₄-associated proteins (Lauterbach *et al.*, 2017b) as well as two C₄-associated transcription factors (Lauterbach *et al.*, 2017a). Gene annotation and pathway assignment can be found in **Supporting Information Table S3**. We listed whether the *S. divaricata* agg. was either in a sister relationship with C₃ species *S. montana* or the C₄ clade. When there were several gene copies or weak bootstrap support, we additionally noted whether our group of interest was in a clade with the C₃ or the C₄ species (for details see **Supporting Information Material & Methods S1**).

Results

Carbon isotope measurements

Salsola divaricata plants growing in the wild on the Canary Islands generally had higher $\delta^{13}\text{C}$ values (-21 to -28; mean -24.44) than those grown from seeds under greenhouse conditions (-22 to -34; mean -27.77). Overall, the $\delta^{13}\text{C}$ values of the *S. divaricata* agg. show a broad range but not a single individual (stressed or not stressed) came close to a C₄-like $\delta^{13}\text{C}$ value of > -16 (**Fig. 2**). We compared $\delta^{13}\text{C}$ values between control, dry and salt treatments within six populations (six individuals per treatment). The Shapiro-Wilk test was used to check for normality of distribution and the Levene's Test for homogeneity of variance. Excluding outlier 198IIr of population 198, all treatment groups of all populations were normally distributed. All populations had homogeneous variances except population 188. Since not all assumptions for an ANOVA test were met we chose the Kruskal-Wallis rank sum test to compare treatments within and between populations. In the control there was no significant difference of $\delta^{13}\text{C}$ values among populations (Kruskal-Wallis chi-squared = 5.1081, df = 5, p-value = 0.40) and the same was found for the drought treatment (Kruskal-Wallis chi-squared = 8.61, df = 5, p-

value = 0.13). In the salt treatment there was a significant difference of $\delta^{13}\text{C}$ values among populations (Kruskal-Wallis chi-squared = 15.47, df = 5, p-value = 0.009), which was due to significantly higher $\delta^{13}\text{C}$ values in salt treated individuals of populations 191 compared to 182 and 194. We tested for differences of carbon isotope values between treatments within populations of the *Salsola divaricata* agg. and found that there was a significant difference for populations 182 (Kruskal-Wallis chi-squared = 7.94, df = 2, p-value = 0.019), 191 (Kruskal-Wallis chi-squared = 10.84, df = 2, p-value = 0.004), and 198 (Kruskal-Wallis chi-squared = 8.84, df = 2, p-value = 0.012). However, there was no significant difference among the three treatments for populations 184 (Kruskal-Wallis chi-squared = 2.33, df = 2, p-value = 0.31), 188 (Kruskal-Wallis chi-squared = 0.78, df = 2, p-value = 0.68), and 194 (Kruskal-Wallis chi-squared = 2.27, df = 2, p-value = 0.32) (**Fig. 3**). After grouping $\delta^{13}\text{C}$ values according to island, significant differences were found among islands (ANOVA: df = 6, F value = 4.08, p-value = 0.001) due to differences between La Gomera and the mainland (SW Morocco), as well as between Lanzarote resp. Tenerife and the mainland. All other differences were not statistically significant. We found that the populations of *S. verticillata*, *S. deschaseauxiana* and *S. gymnomaschala* on the Moroccan mainland often had higher $\delta^{13}\text{C}$ values than the populations of *S. divaricata* on the Canary Islands (the non-stressed control groups that are regularly watered, **Supporting Information Fig. S1**). However, due to very different sample sizes further statistical tests including all populations were inconclusive.

Overall, these results show a broad plasticity of $\delta^{13}\text{C}$ values among populations associated with source population and growth conditions well within the range typical of C_3 species. The significantly higher $\delta^{13}\text{C}$ values of salt treated individuals indicate that salinity rather than drought affects the $\delta^{13}\text{C}$ in *Salsola divaricata* agg. and could be responsible for the occasional observation of relatively high $\delta^{13}\text{C}$ values of our samples collected in the wild close to the shoreline.

Fig. 2: Distribution of $\delta^{13}\text{C}$ (‰) of 195 samples of the *Salsola divaricata* agg., of these 30 were collected from plants growing in the wild and 165 from plants in cultivation at the Botanical Garden Mainz. This includes 36 individuals in dry treatment and 36 in salt treatment, all others are in the control group. See **Table S1** for information on populations included.

Fig. 3: Comparison of carbon isotope values ($\delta^{13}\text{C}$ (‰)) between treatments within populations (P) of the *Salsola divaricata* agg. Significant differences were found for populations 182 from La Gomera (a; Kruskal-Wallis chi-squared = 7.94, df = 2, p-value = 0.02), 191 from Fuerteventura (d; Kruskal-Wallis chi-squared = 10.84, df = 2, p-value = 0.004), and 198 from Gran Canaria (f; Kruskal-Wallis chi-squared = 8.84, df = 2, p-value = 0.01). No significant differences were found between the three treatments for 184 from Lanzarote (b; Kruskal-Wallis chi-squared = 2.33, df = 2, p-value = 0.31), 188 from La Graciosa (c; Kruskal-Wallis chi-squared = 0.78, df = 2, p-value = 0.68), and 194 from Fuerteventura (e; Kruskal-Wallis chi-squared = 2.27, df = 2, p-value = 0.32).

CO₂ compensation point measurements

The CO₂ compensation points of the four species of the *Salsola divaricata* agg. lie between those of the C₃ (*Salsola webbii*) and C₄ species (*Salsola oppositifolia*) as expected (between 20 and 30 $\mu\text{mol CO}_2 \text{ mol}^{-1}$, **Fig. 4a**). With 19.56 and 21.22 $\mu\text{mol CO}_2 \text{ mol}^{-1}$, respectively, the mean values of *S. deschaseauxiana* and *S. verticillata* were slightly lower than those of *S. divaricata* and *S. gymnomaschala* (25.14 and 26.75 $\mu\text{mol CO}_2 \text{ mol}^{-1}$ respectively). When looking at individual measurements we again observed a broad range from 17 to 40 $\mu\text{mol CO}_2 \text{ mol}^{-1}$ with one outlier at 9 $\mu\text{mol CO}_2 \text{ mol}^{-1}$ (**Supporting Information Fig. S2**). Populations growing on the more arid Islands such as Lanzarote and Fuerteventura or in Morocco did not show lower CO₂ compensation points. Due to varying values within each population (**Supporting Information Fig. S2**) and the relatively small sample sizes (one to two individuals per population) we did not conduct further statistical analysis.

Fig. 4: a) CO₂ compensation points (Γ in $\mu\text{mol CO}_2 \text{ mol}^{-1}$) of the separate species of the C₂ *Salsola divaricata* agg. (Sd_C2 = *Salsola divaricata*, Sg_C2 = *S. gymnomaschala*, Sv_C2 = *S. verticillata*, Sx_C2 = *S. deschaseauxiana*) compared to the values of C₃ species *Salsola webbii* (Sw_C3) and C₄ species *Salsola oppositifolia* (So_C4). **b)** PEPC activity [$\mu\text{mol min}^{-1} \text{ mg}^{-1}_{\text{chlorophyll}}$] of the separate species of the C₂ *Salsola divaricata* agg. compared to C₄ *S. oppositifolia* and C₃ *S. webbii*.

PEPC activity measurements

First, we compared the PEPC activity of the separate species of the *Salsola divaricata* agg. to the values of the closely related C₃ species *Salsola webbii* and C₄ species *Salsola oppositifolia*. C₃ (n=1, 30 $\mu\text{mol min}^{-1} \text{ mg}^{-1}_{\text{Chlorophyll}}$) and C₄ values (n=2, mean 216 $\mu\text{mol min}^{-1} \text{ mg}^{-1}_{\text{Chlorophyll}}$) were as expected clearly distinguishable (**Fig. 4b**). The values of the C₂ species were closer to

those of the C₃ species and much lower than the C₄ species (*Salsola divaricata*, n=82, mean activity 33 $\mu\text{mol min}^{-1} \text{mg}_{\text{Chlorophyll}}^{-1}$; *S. gymnomaschala*, n=10, mean 46.8 $\mu\text{mol min}^{-1} \text{mg}_{\text{Chlorophyll}}^{-1}$; *S. verticillata*, n=7, mean 37.4 $\mu\text{mol min}^{-1} \text{mg}_{\text{Chlorophyll}}^{-1}$; *S. deschaseauxiana*, n=6, mean 27.6 $\mu\text{mol min}^{-1} \text{mg}_{\text{Chlorophyll}}^{-1}$; **Fig. 4b**). We used a Kruskal-Wallis test to determine if statistically significant differences exist between C₂ species (Kruskal-Wallis chi-squared = 9.94, df = 3, p-value = 0.019). This significant difference was observed due to the values of *S. divaricata* on the Canary Islands having a median of 30.05 $\mu\text{mol min}^{-1} \text{mg}_{\text{Chlorophyll}}^{-1}$ and *S. gymnomaschala* in Morocco having a median of 48.32 $\mu\text{mol min}^{-1} \text{mg}_{\text{Chlorophyll}}^{-1}$. Due to the high level of variation within species we took a closer look at 14 populations of *S. divaricata* and observed a significant difference between them (Kruskal-Wallis chi-squared = 34.64, df = 13, p-value = 0.001, see also **Fig. 5a**). When grouped according to island, we found a significant difference (Kruskal-Wallis chi-squared = 22.199, df = 5, p-value = 0.0005) which is due to the difference in values between populations on Gran Canaria and Lanzarote, as well as populations on La Graciosa and Fuerteventura and on La Graciosa and Gran Canaria (**Fig. 5b**). We did not find a significant difference between treatments in the greenhouse in *S. divaricata* (Kruskal-Wallis chi-squared = 1.21, df = 2, p-value = 0.55). Again, we observe a high variation of PEPC activity values within populations and islands.

Fig. 5: Boxplots of PEPC activity [$\mu\text{mol min}^{-1} \text{mg}_{\text{Chlorophyll}}^{-1}$] of *Salsola divaricata* Moq. **a)** Values grouped by population (P). **b)** Values grouped by island; F = Fuerteventura, GC = Gran Canaria, L = Lanzarote, LGo = La Gomera, LGra = La Graciosa, T = Tenerife.

Phylogenomic analyses

The raw reads for the newly generated transcriptomes are available from the NCBI Sequence Read Archive (**Table 2**). We obtained 991 final MO orthologs that included all 17 taxa with alignments of lengths between 315 and 6621 bp. The concatenated matrix comprises 1,427,449 aligned columns with overall matrix occupancy of 86% (see **Supporting Information Table S2** alignment occupancy per taxon).

The ASTRAL species tree and the concatenated RAxML tree (**Supporting Information Fig. S3**) had similar topologies with most nodes receiving the maximum support (BS = 100%; LPP = 1). *Caroxylon vermiculatum* was recovered as sister to all other Salsoleae, followed by a grade formed by *Salsola webbii*, *Salsola genistoides*, *Kali collina*, and *Salsola montana*. *Salsola divaricata* and *Salsola verticillata* form a clade placed as sister of the clade composed of *Anabasis articulata*, *Salsola soda*, *Salsola oppositifolia*, *Halogeton*, *Hammada*, and *Holoxylon*. The only difference between the two topologies is that *Anabasis articulata* and *Salsola soda* were sisters with maximum support in the RAxML tree but formed a grade with lower support (LPP = 0.74) in the ASTRAL tree.

Concordance analyses (**Supporting Information Fig. S3 and S4**) overall showed high support for the monophyly of Salsoleae and the *Salsola divaricata* agg., while signaling conflict for the remaining taxa. The placement of *Salsola genistoides* had low support with only 261 out of 794 informative gene trees being concordant (ICA = -0.45) and high QS score (0.22/0/0.98) but also suggesting an alternative topology. The clade composed of *Salsola divaricata* + *Salsola verticillata* had strong support with 808 out of 913 gene trees being concordant (ICA = 0.71) and maximum QS support (1/-/1; i.e., all sampled quartets supported that branch). However, the two samples of *Salsola divaricata* showed signals of alternative topologies with only 332 out of 900 gene trees supporting their sister relationship (ICA = 0.56) and QS counter support (-0.25/0/1). The sister relationship of *Salsola divaricata* + *S. verticillata* and the remaining Salsoleae had low support with only 178 out of 705 gene trees being concordant (ICA = 0.29) and also low QS support (0.085/0/1 [ASTRAL]; (0.11/0/1) [RAxML]) suggesting an alternative topology. The placement of *Anabasis articulata* and *Salsola soda*, which varied between RAxML and ASTRAL trees, also had low gene tree and QS support with signals of alternative topologies. The clade composed of *Salsola oppositifolia*, *Halogeton*, *Hammada*, and *Holoxylon* was supported only by 280 out of 692 gene trees (ICA = 0.24), but had maximum QS support,

while within the clade all relationships have low gene tree and QS support signaling alternative topologies.

The final plastid alignment had 80,903 characters with a matrix occupancy of 70%. The plastid tree largely differed from the nuclear topologies and had most nodes with maximum support (BS = 100; **Fig. 6** and **S5**). *Caroxylon vermiculatum*, *Kali collina*, and *Salsola genistoides* form a well supported clade (BS = 100; QS = 0.56/0/1) that is sister to all Salsoleae. *Salsola webbii* and *Anabasis articulata* form a grade with strong support (BS = 100; QS = 0.6/0/0.99 [*S. webbii*]; QS = 1/-/1 [*A. articulata*]). The remaining species form an unsupported clade (BS = 0.47; QS = -0.0096/0.88/0.68) composed of two subclades. The first subclade (BS = 93; QS = 0.19/0.91/0.78) is composed of *Salsola verticillata* + *Salsola montana* (BS = 100; QS = 0.61/0/1) and the two samples of *Salsola divaricata* (BS = 100; QS = 1/-/0.77). The second subclade (BS = 100; QS = 0.59/0/0.95) has *Salsola soda* as the sister the clade (BS = 100; QS = 1/-/1) composed of *Salsola oppositifolia*, *Halogeton*, *Hammada*, and *Haloxyton*.

Fig. 6: a) Maximum likelihood phylogeny of Salsoloideae inferred from the RAXML analysis of the concatenated 991-nuclear gene supermatrix from the 'monophyletic outgroup' (MO) orthologs. Bootstrap support and local posterior probabilities (BS/LPP) are shown above branches. Nodes with full support (BS =

100/LPP = 1) are not shown. Em dashes (—) denote alternative topology compared to the ASTRAL tree (Supporting Information Fig. S3). Quartet Sampling (QS) scores are shown below branches. QS score: Quartet concordance (QC)/Quartet differential (QD)/Quartet informativeness (QI). Full QS support (1/–/1) not shown. Pie charts represent the proportion of gene trees that support that clade (blue), the proportion that support the main alternative bifurcation (green), the proportion that support the remaining alternatives (red), and the proportion (conflict or support) that have < 50% bootstrap support (gray). Branch lengths are proportional to the number of substitutions per site (scale bar on the bottom). b) Cladogram of Salsoloideae inferred from IQ-TREE analysis of concatenated complete and partial plastomes. QS scores and BS support are shown above and below branches, respectively. See Supporting Information Fig. S5 for phylogram. c) Best maximum pseudo-likelihood species network inferred with PhyloNet. Red and blue curved branches indicate the minor and major edges, respectively of hybrid nodes. Numbers next to curved branches indicate inheritance probabilities for each hybrid node.

Assessment of hybridization

The model selection showed that a network was always a better model than a bifurcating tree (Table 3). A network with three hybridization events is the best model (AICc=37875.93) compared to all others, including the best bifurcating model (RAxML; AICc=39547.83). The best network had three reticulation events (Fig. 6c). First, the *Salsola divaricata* and *Salsola verticillata* clade was a product of a hybridization event between *Salsola soda* (C₄) (inheritance probability $p_i = 0.346$) and *Salsola montana* (C₃) ($p_i = 0.654$). The second reticulation event showed that the clade formed by *Salsola oppositifolia*, *Halogeton*, *Hammada*, and *Haloxylon* (all C₄) is the product of a hybridization event between *Salsola soda* (C₄) ($p_i = 0.846$) and the ancestor lineage of *Salsola divaricata* + *Salsola verticillata* (C₂) ($p_i = 0.154$). These two clades were also recovered as hybrids in the other networks with one, two and three reticulation events with similar parental lineages and inheritance probabilities (Supporting Information Fig S6). The third event is a deeper reticulation within *Salsoleae* which showed that most species of the group (excluding *Caroxylon*) were a product of an ancient hybridization event between *Salsola genistoides* ($p_i = 0.212$) and the sister lineage of *Salsola genistoides* + *Salsola webbii* ($p_i = 0.788$). The HyDe analysis of all possible triples at the individual level resulted in 1,680 hybridization tests, of which 298 triples were significant. The significant triples showed that 12 out of 13 individuals of *Salsoleae* are involved in multiple hybridization events, which is mostly in agreement with the nested reticulation events detected with Phylonet. The admixture parameter (γ) ranged from 0.013 to 0.986 (average 0.458). These results indicate frequent hybridization within *Salsoleae*. Additionally, Hyde also identified *Eokochia saxicola* as a hybrid (Supporting Information Table S3, Fig. S7).

Individual analysis of genes or gene families involved in photosynthesis or photorespiration

The number of sequences in the 50 alignments ranged from 7 to 65 due to variation in the number of accessions and gene copies (**Supporting Information Table S3**). Sequence lengths ranged from an average minimum of 420 (255 to 867) to an average maximum of 1,467 bp (462 to 3,126) with an average length of 1,168 in all sequences. Summarizing all genes, *Salsola divaricata* agg. appeared 22 times as sister to C₃ *S. montana* and 26 times as sister to the C₄ clade regardless of bootstrap support (**Supporting Information Table S3**). In three alignments *S. divaricata* agg. was missing. When multiple copies per gene were recovered for *S. divaricata* agg, they were often non-monophyletic, but instead separated in clades with copies from the C₃ and/or C₄ species. In these cases, sequences of the *S. divaricata* agg. appeared 21 times with C₃ and 26 times with C₄ species. Separated by pathway assignment, the *S. divaricata* agg. was 16 times sister to C₃ (11 times in clade with C₃) and 15 times sister to C₄ (17 times in clade with C₄) for C₄ genes (independent from bootstrap support). When incorporating bootstrap support and discarding everything below 70, the numbers shrink to 10 times sister to C₃ (8 times in clade with C₃) and 10 times sister to C₄ (13 times in clade with C₄) for C₄ genes. For the two C₄-associated transcription factors, a sister relationship of *S. divaricata* agg. to the C₄ clade is supported for SHORTROOT (BS = 86). The sister relationship to the C₄ clade is not well supported for BEL1-like homeodomain 7 (BS =17 sister to C₄, 57 in clade with C₄). For the genes assigned to the Glyoxylate cycle *S. divaricata* agg. appears with several copies which are grouped with high support with either C₃ or C₄ (BS > 80). One locus is missing in the *S. divaricata* agg. and *S. montana*. *Salsola divaricata* agg. is also missing in the alignments for the RuBisCO subunits. In the trees for photorespiration-associated genes many species are represented with several copies, even though during the transcriptome processing putative genes were inferred to remove isoforms and assembly artifacts (i.e. misassemblies and chimeras). This is also true for *S. divaricata* agg. which is also rarely monophyletic. The sister relations of *S. divaricata* agg. to the C₃ species are not well supported (5 times with BS < 70), but clade memberships with the C₃ species are (9 times, BS > 70). Sister relations to the C₄ clade are well supported in six of the nine times this occurred, with supported shared clade membership in seven of ten cases. Just like the *S. divaricata* agg. the C₄ clade is also not monophyletic in most cases. Clade assignment for all 50 genes is listed in **Supporting Information Table S3** and the individual trees are shown in the **Supporting Information Fig S8**.

Discussion

In this study we aim to distinguish two alternative hypotheses concerning the origin of C₃-C₄ intermediacy of the *Salsola divaricata* agg. The model of C₄ evolution (e.g., Sage, 2004) interprets C₂ as an evolutionary stepping stone to fully functional C₄ photosynthesis. *Salsola divaricata* is currently classified as a C₂ Type I according to Sage *et al.* (2014). A strong selective pressure towards an increase of PEPC activity should be expected under more stressful (more carbon deficient) conditions in the more arid part of the distribution area (Lanzarote, Fuerteventura, Morocco) and a transition to the C₂ Type II or even C₄-like intermediate type, resulting in a reduction of photorespiration (Sage *et al.*, 2014). In case of a hybrid origin of C₂ in the *Salsola divaricata* agg. caused by a hybridization event at some point in the evolutionary history of the Salsoleae, which is the alternative working hypothesis, we should see an independent segregation of C₄-traits (Brown & Bouton, 1993; Oakley *et al.*, 2014); and, a phylogenetic signal indicating a hybridization event involving the *Salsola divaricata* lineage.

Carbon isotope measurements depict a stress reaction in the *S. divaricata* agg.; but without occurrence of a more C₄-like photosynthesis in populations growing in drier environments

Although samples collected in the wild had slightly higher $\delta^{13}\text{C}$ values than those sampled in our greenhouse (stressed or control), there is still a clear difference to typical values of C₄ species. None of the samples showed a $\delta^{13}\text{C}$ value typical for a C₄-like or a C₄ plant which would be around -9 to -16 ‰ (Sage, 2016); instead all samples had values falling within the range typical for C₃ species which would be around -22 to -32 ‰ (Sage, 2016). In C₃ plants the $\delta^{13}\text{C}$ values of leaves depend on a number of factors including stomatal conductance and capacity to capture CO₂ in photosynthesis which is dependent on light intensity, growth temperature, photochemistry and capacity for carbon assimilation. The plants growing in the greenhouse are under less stress for potential water loss than field conditions due to lower light intensity, and temperature being more moderate than some field conditions. The results suggest a greater stomatal limitation on photosynthesis in the field grown plants, which results in a low intercellular CO₂ concentration. This will change the discrimination against the heavier C isotope since RuBisCO will capture more ¹³CO₂ resulting in slightly higher $\delta^{13}\text{C}$ values (von Caemmerer, 1992).

We observed a broad plasticity of $\delta^{13}\text{C}$ values. Populations on the Moroccan mainland often have higher values than the populations on the Canary Islands; but there are many exceptions

in populations on the more arid Islands of Fuerteventura and La Graciosa as well as on the more humid Islands of Gran Canaria, Tenerife and La Gomera. There does not seem to be a clear pattern correlated with the general climatic conditions on the Islands. Therefore, we assume that the observed variation is due to local, microclimatic differences. In any case, there are no indications of populations exhibiting C₄-like photosynthesis on the basis of the $\delta^{13}\text{C}$ values. *Salsola divaricata* plants from all populations grown for several years under salt stress in the greenhouse showed clear morphological differences in comparison to the control plants. They had more succulent leaves, did not grow to the same height as their counterparts in the control group and most individuals in the salt group flowered earlier. In three of the six tested populations, La Gomera (182), Fuerteventura (191), and Gran Canaria (198), this long-term salt stress was reflected in a significantly higher $\delta^{13}\text{C}$ value. The three other populations from Lanzarote, La Graciosa and Fuerteventura had slightly, but not significantly higher, values for the salt treatment compared to the control group. This suggests *Salsola divaricata* can adapt to salt stress by increasing water storage and decreasing stomatal conductance and it explains some of the $\delta^{13}\text{C}$ value variation found in the wild as salinity certainly varies among sites.

C₃-C₄ intermediate CO₂ compensation points for *S. divaricata* agg. supports a stable C₂-state with high plasticity

The CO₂ compensation points of the *S. divaricata* agg. are well within the range of other C₂ species (Sage *et al.*, 2014 and ref. therein) and they are significantly lower than the values of C₃ species *S. webbii* and significantly higher than those of C₄ species *S. oppositifolia*. There is no evidence for constantly lower Γ values in populations that originated from more arid regions, although individuals of *S. verticillata* and *S. deschaseauxiana* mostly show somewhat lower values, many individuals from similar climatic environments (e.g. *S. gymnomaschala* or individuals of *S. divaricata* from Fuerteventura or Lanzarote) show higher values which screws a possible relation of climate and lower Γ values. Sage *et al.* (2014) outlined that a Γ value below 10 $\mu\text{mol CO}_2 \text{ mol}^{-1}$ characterizes the transition from C₂ Type I to C₂ Type II and represents an evolutionary progression towards C₄. An accessory C₄ metabolic cycle is present in C₂ Type II plants and was so far only observed in several species of *Flaveria* and *Mollugo verticillata* (Edwards & Ku, 1987). None of the Γ measurements of 53 individuals of the *Salsola divaricata* agg. shows an indication of an accessory C₄ metabolic cycle and a significantly higher carboxylation by PEPC.

So far the analysed traits seem to be independent between populations and islands and show a high plasticity. On Lanzarote for example, populations of *S. divaricata* have comparably high Γ values and medium to low carbon isotope values compared to other populations.

PEPC activity does not relate to Γ values or carbon isotope values in *S. divaricata* agg.

The PEPC activity of the *S. divaricata* agg. is similar to the C₃ species *S. webbii*. Even though there was a high standard deviation within C₄ *S. oppositifolia*, it is evident that none of the C₂ individuals comes even close to a C₄-like value. The activity of PEPC for *S. verticillata* and *S. deschaseauxiana* are slightly lower than those for *S. divaricata*; therefore, their slightly lower Γ values and slightly higher carbon isotope values are not due to increased CO₂ fixation by an optimized PEPC. Thus, *S. verticillata* and *S. deschaseauxiana* do not seem to be closer to a more C₄-like state than *S. divaricata* or *S. gymnomaschala*.

The PEPC activity in the *S. divaricata* agg. is highly variable and shows a lack of correlation with carbon isotope values and CO₂ compensation points, as discussed above for *S. verticillata*. We would expect a higher PEPC activity in the salt treatment or the more arid locations e.g. Lanzarote or Fuerteventura, however that was not the case. Although there are statistically significant differences between islands, it is not possible to predict a value for PEPC activity given the location since there is generally a large overlap of values. The differences between populations could not be explained by the climatic context of the parent plant in the wild and they were all treated the same in the greenhouse. We could not detect a correlation between PEPC activity and the previously discussed traits, supporting the independent distribution of the traits.

The independent variation in three photosynthetic traits (carbon isotope value, CO₂ compensation point and PEPC activity) does not support a directed selection towards C₂ Type II or C₄-like photosynthesis in any of the populations studied, not even those growing in semi-desert conditions. In contrast, our trait observations are more in line with the results of hybridization experiments (Brown & Bouton, 1993; Oakley *et al.*, 2014; Kadereit *et al.*, 2017) which supports the hypothesis of a hybrid origin of the *Salsola divaricata* agg. Summarizing the physiology of advanced generations of interspecific C₃ and C₄ hybrids, Brown & Bouton (1993) stated that the 'correlation among photosynthetic traits were low' which indicates 'a high degree of independence, both genetic and physiological, among C₄ traits'. Like in our study, the hybridization experiment of C₃ and C₄ *Atriplex* (Oakley *et al.*, 2014) showed a broad range of Γ values (in nine F₂ individuals from 25-45 $\mu\text{mol CO}_2 \text{ mol}^{-1}$; and one C₄-like value).

Brown & Bouton (1993) hypothesize that some of the photosynthetic traits measured can be more C₄-like due to non-disturbance of the C₃ metabolism, such as Γ , while others mirror the physiological C₃ default condition, such as enzyme activity.

First phylogenomic evidence of a hybrid origin of the *S. divaricata* agg.

The Salsoleae species tree shows a high level of conflict including the *S. divaricata* agg, despite high bootstrap support. The assessment of hybridization events using PhyloNet and HyDe showed multiple hybridization events within Salsoleae in which the *Salsola divaricata* agg. was likely involved. The incomplete sampling of Salsoleae does not allow us to identify the exact parental lineages; but a hybrid origin of the *Salsola divaricata* agg. was highly supported. The network that was recovered most often shows a possible ancient hybridization event involving an ancestor of C₄ *Salsola soda* and the C₃ proto-kranz *Salsola montana* giving rise to the *Salsola divaricata* agg. clade. This is the first genomic evidence that a C₃-C₄ intermediate species is the result of (ancient) hybridization between a C₄ and a C₃ lineage. The second reticulation event at a later time indicates a possible hybridization event involving the ancestor of *Salsola divaricata* + *Salsola verticillata* and the C₄ *Salsola soda* giving rise to the C₄ clade formed by *Salsola oppositifolia*, *Halogeton*, *Hammada*, and *Haloxydon*. This might indicate that this C₄ lineage could have inherited C₄ or antecedent traits from a parental lineage giving them an adaptive advantage which might have influenced their photosynthesis pathway, e.g. adaptations in photosynthesis or photorespiration associated enzymes or Kranz-cells. We also found hybridization events in the aforementioned two clades in the other networks with similar parental lineages, supporting the evidence that these lineages were involved in hybridization events. This could indicate either an incomplete reproductive barrier in the early lineages and recurrent backcrossing or incidences of horizontal gene transfer. We have to keep in mind though that our sampling is limited and that other lineages not sampled in the C₄ clade might also play a role (see Schüssler *et al.*, 2017 for a plastid tree of the Salsoloideae).

Another explanation for the observed picture could be horizontal gene transfer (HGT). Dunning *et al.* (2019) showed that an individual of *Alloteropsis semialata* (Paniceae, Poaceae) contained at least 59 genes which were acquired via horizontal gene transfer (HGT) from approximately nine different grass donors often growing in close proximity. The mechanism is not determined as of yet though they assume allopolyploids which backcrossed with diploids, occasional interspecific cell-to-cell contact through root-to-root interaction as well as illegitimate pollination could be possibilities. Of these, HGTs functional genes included photosynthetic

genes alongside genes incorporated in disease resistance and abiotic stress response (Dunning *et al.*, 2019). Key enzymes of the C₄ pathway were thus acquired by *Alloteropsis* which led to a switch to C₄ photosynthesis (Christin *et al.*, 2012). There are other examples of HGT occurring in green plants which can lead to adaptive innovations (reviewed in Chen *et al.*, 2021). Just like grasses, *Salsola* is wind pollinated and a similar occurrence of illegitimate pollination as described in Christin *et al.* (2012) and Roalson (2012) is not ruled out. However, the only *Salsola* currently growing in close proximity to the *Salsola divaricata* agg. is *S. oppositifolia*.

Trees of photosynthetic genes supports the hybrid origin hypothesis and suggests candidate genes for further study

A closer look at c. 50 gene trees of genes or gene families involved in photosynthesis or photorespiration revealed the same overall pattern, namely incongruence regarding the position of the *Salsola divaricata* agg. which in most cases either groups with the C₃ *S. montana* (also the case in the plastid dataset) or with the C₄ species. This pattern could be explained by hybridization of parental C₃ and C₄ lineages giving rise to an allopolyploid *S. divaricata* agg. with an intermediate expression of photosynthetic traits. The populations of the *S. divaricata* agg. studied here are all tetraploid (D. Tefarikis, unpublished flow cytometry data). The ploidy level of *S. montana* is unknown. In the C₄ clade, ploidy levels from di-, tetra- and octoploid are present (Kew C-value database and D. Tefarikis, unpublished flow cytometry data).

The kranz-like salsoloid leaf type of the *S. divaricata* agg. (Schüssler *et al.*, 2017: Fig. 7), for example, might occur as in the case of the gene SHORTROOT, a C₄ associated transcription factor which influences the development of kranz cells (Kelly *et al.*, 2017, Slewinski *et al.* 2014), with expression of the parental C₄ copy. Studying transcriptome profiles of Salsoleae and Camphorosmeae leaves, respectively, Lauterbach *et al.* (2017a) and Siadjeu *et al.* (2021) revealed a high expression of SHORTROOT in C₂ species. In case of PEPC two copies were sequenced and for both the *S. divaricata* agg. group with the C₃ species supporting the C₃-like PEPC activity data found in this study.

Conclusions

The phylogenetic signal in our trees, gene trees and networks indicate hybridization events gave rise to the *Salsola divaricata* agg. Furthermore, the broad but independent segregation of three photosynthetic traits in a C₃-C₄ intermediate range without indication of selection for a more C₄-like expression supports a hybrid origin of this C₂ clade. Overall the photosynthetic traits

seem plastic and genetically fixed stress reactions are not prominent among the populations studied. The *Salsola divaricata* agg. is well adapted to a broad range of climatic conditions including mesic to arid coastal habitats and also to salinity. Part of the success of the species aggregate might be the photorespiratory pump acquired through hybridization with a C₄ lineage in the past. We therefore conclude that this C₂ species aggregate does not represent an evolutionary stepping stone towards C₄; rather the more likely explanation is a hybrid origin. However, a broader sampled phylogenomic study is needed to reveal the parental lineages. We propose that hybridization events might also have influenced the evolutionary history of other C₂ lineages, and that in particular those genera on which the model of C₄ evolution was based should be tested.

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Author Contributions

GK designed the study. DT and GE (carbon isotope values) measured the trait data and DT analysed them. DT, DMB and YY conducted the phylogenomic analyses and hybridization networks. DMB prepared the figures. DT, GK and DMB wrote the first draft. All authors contributed to the discussion of the results and the final version of the manuscript.

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Data Availability Statement

The datasets analyzed for this study can be found in the NCBI Sequence Read Archive (SRA) (see **Table 2** for SRA accession numbers).

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The following Supporting Information is available for this article:

Fig. S1: Carbon isotope values ($\delta^{13}\text{C}(\text{‰})$) of different populations (P) of the *Salsola divaricata* agg. on the Canary islands and Morocco

Fig. S2: CO₂ compensation points (Γ in $\mu\text{mol CO}_2 \text{ mol}^{-1}$) of populations (P) of the *Salsola divaricata* agg.

Fig. S3: a) Cladogram of Salsoloideae inferred from the Maximum likelihood analyses of the concatenated 991-nuclear gene supermatrix. b) Cladogram of Salsoloideae inferred from the ASTRAL analyses of 991 nuclear genes trees.

Fig. S4: A) RAxML cladogram of Salsoloideae inferred from the concatenated 991-nuclear gene supermatrix. B) ASTRAL Cladogram of Salsoloideae inferred from 991 nuclear genes trees.

Fig S5: Maximum likelihood phylogeny of Salsoloideae s.l. inferred from the IQ-TREE analysis of complete and partial plastomes.

Fig. S6: Maximum pseudo-likelihood species network inferred with PhyloNet with up to five hybridization events.

Fig. S7: HyDe boxplot of the distribution of the admixture parameters (γ) from the 298 significant tests.

Fig. S8: Maximum likelihood cladograms of the 50 photosynthetic gene trees. Bootstrap support (BS) values are shown above the branches.

Table S1: Populations (named by living collection number in the Botanical Garden Mainz; see Fig. 1) included in the carbon isotope measurements, CO₂ compensation point measurements and PEPC activity measurements, including sampling location and collector.

Table S2: Gene and character occupancy of the concatenated matrix.

Table S3: HyDe results from the 298 significant tests.

Materials and Methods S1: More detailed description of methods of CO₂ compensation point, PEPC activity measurement, Transcriptome processing and nuclear phylogenetic analyses, Assessment of hybridization, Plastome assembly and phylogenetic analysis, and Analysis of photosynthetic gene trees.

References Materials and Methods S1

Tables

Table 1: List of the 17 samples in the transcriptome phylogeny; with photosynthesis type, ploidy (Kew C value database 2018) and NCBI Sequence Read Archive (SRA) accession number.

Species	Tribe	Ploidy	Photosynthesis type	SRA accession No.
<i>Anabasis articulata</i> Choul. ex Pomel	Salsoleae	NA	C ₄	SRR6435311
<i>Halogeton glomeratus</i> (M. Bieb.) C.A. Mey.	Salsoleae	2x	C ₄	SRR1503502
<i>Haloxylon ammodendron</i> (C.A. Mey.) Bunge	Salsoleae	2x	C ₄ (C ₃ cotyledon)	SRR1697346
<i>Hammada scoparia</i> (Pomel) Iljin	Salsoleae	2x	C ₄ (C ₃ cotyledon)	ERR2060287
<i>Kali collina</i> Akhani & Roalson	Salsoleae	2x	C ₄	SRR6435349
<i>Salsola divaricata</i> Moq. (Pop.184)	Salsoleae	4x	C ₂	ERR2060298
<i>Salsola divaricata</i> Moq. (Pop.198)	Salsoleae	4x	C ₂	ERR2060294
<i>Salsola genistoides</i> Juss. ex Poir.	Salsoleae	4x	C ₃	SRR14783909
<i>Salsola montana</i> Litv.	Salsoleae	NA	C ₃ proto-kranz	SRR14783908
<i>Salsola oppositifolia</i> Desf.	Salsoleae	8x	C ₄	ERR2060302
<i>Salsola soda</i> L.	Salsoleae	2x	C ₄ (C ₃ cotyledon)	SRR3544552
<i>Salsola verticillata</i> Schousb. (Pop.171)	Salsoleae	4x	C ₂	SRR14783907
<i>Salsola webbii</i> Moq.	Salsoleae	4x	C ₃	ERR2060308
<i>Caroxylon vermiculatum</i> (L.) Akhani & Roalson	Caroxyloneae	NA	C ₄	SRR6435345
<i>Bassia scoparia</i> (L.) A.J. Scott	Camphorosmeae	2x	C ₄	ERR364385
<i>Eokochia saxicola</i> (Guss.) Freitag & G.Kadereit	Camphorosmeae	NA	C ₃	SRR6435348
<i>Beta vulgaris subsp. vulgaris</i> L.	Beteae	2x	C ₃	SRX335625

Table 2: Model testing for 17 taxa and 991 gene trees. Comparing log likelihoods of the trees and networks. The best model is the network with three hybridization events from PhyloNet (best MPL run allowing four hybridization events). k = No. branch lengths + hybridization probabilities; h = number of hybridization events.

tree/network	ln L (log likelihood)	k	h	AIC	deltaAIC	AICc	deltaAICc	BIC	deltaBIC
astral_calgtp_rob	-19754.54543	31	N/A	39571.091	1698.041	39573.130	1697.190	39722.951	1668.649
raxml_calgtp_rob	-19741.89558	31	N/A	39545.791	1672.742	39547.830	1671.891	39697.651	1643.349
iqtree_plastome_calgtprob	-22836.08408	31	N/A	45734.168	7861.119	45736.207	7860.268	45886.028	7831.726
phylonet_mpl_1hyb[1]	-19451.79848	31	1	38965.597	1092.547	38967.636	1091.696	39117.457	1063.155
phylonet_mpl_1hyb[2]	-19605.00833	33	1	39276.017	1402.967	39278.323	1402.383	39437.674	1383.372
phylonet_mpl_1hyb[3]	-19624.56237	33	1	39315.125	1442.075	39317.431	1441.491	39476.782	1422.480
phylonet_mpl_2hyb	-19435.04197	35	2	38940.084	1067.034	38942.674	1066.734	39111.539	1057.237
phylonet_mpl_3hyb	-19276.12872	37	3	38626.257	753.208	38629.147	753.208	38807.510	753.208
phylonet_mpl_4hyb	-18899.52481	37	3	37873.050	0.000	37875.940	0.000	38054.302	0.000
phylonet_mpl_5hyb	-19283.48635	41	5	38648.973	775.923	38652.512	38652.512	38849.820	38849.820