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On the hybrid origin of the C_2 *Salsola divaricata* agg. (Amaranthaceae) from C_3 and C_4 parental lineages

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Summary

- C_2 photosynthesis is characterised using recapturing photorespiratory CO_2 by RuBisCo in Kranz-like cells and is therefore physiologically intermediate between C_3 and C_4 photosynthesis. C_2 can be interpreted as an evolutionary precursor of C_4 and/or as the result of hybridisation between a C_3 and C_4 lineage.
- We compared the expression of photosynthetic traits among populations of the *Salsola divaricata* agg. (C_2) 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 C_4-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 hybridisation analyses we tested for the hybrid origin of the *Salsola divaricata* agg.
- We observed distinct independent variation of photosynthetic traits within and among populations and no clear evidence for selection towards C_4-like trait expression in more stressful habitats or treatments. We found reticulation and gene tree incongruence in Salsoleae supporting a putative hybrid origin of the *Salsola divaricata* agg.
- C_2 photosynthesis in the *Salsola divaricata* agg. combines traits inherited from its C_3 and C_4 parental lineages and seems evolutionarily stable, possibly well adapted to a wide climatic amplitude.

Introduction

In current models of C_4 evolution, the C_3-C_4 intermediate phenotypes (including C_2 plants) are interpreted as a transitional evolutionary link between the ancestral C_3 photosynthesis pathway and the derived C_4 pathway and showcase the complexity of the numerous structural, genetic, and functional changes necessary to establish a functioning C_4 pathway (Bräutigam & Gowik, 2016; Schlüter & Weber, 2016). There are *c.* 50 known species with intermediate C_3–C_4 traits, some of which do not share a common ancestor with one of the more than 60 independent C_4 lineages, and some of which represent lineages up to 20 or even 30 million years (Myr) in their crown age (Sage *et al.*, 2018). The fact that we observe these intermediate phenotypes in nature repeatedly implies that they can be evolutionary stable (Lundgren, 2020). Their phenotypic diversity has been classified into four photosynthetic categories of C_3–C_4 intermediacy: proto-Kranz, C_2 type I, C_2 type II and C_4-like (Sage *et al.*, 2014). C_2 photosynthesis has been repeatedly interpreted as a crucial stepping stone towards C_4 photosynthesis (Edwards, 2019). In C_2 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 CO_2 is recaptured by RuBisCo (Schulze *et al.*, 2016). Bräutigam & Gowik (2016) proposed that once the photorespiratory CO_2 pump is active, establishing the C_4 cycle is inevitable. According to this model, a strong selective pressure towards an increase of phosphoenolpyruvate carboxylase (PEPC) activity in C_2 species should be expected under carbon-deficient conditions (Bräutigam & Gowik, 2016).

A possible alternative, but not mutually exclusive hypothesis, is the origin of C_3–C_4 intermediates through hybridisation (Monson & Edwards, 1984). Experimental hybrids of C_3 and C_4 species reveal similar phenotypes as naturally occurring C_3–C_4 intermediate species and a segregating of photosynthetic traits in F_{2+} generations (Björkman *et al.*, 1969; Holaday *et al.*, 1985; Cameron *et al.*, 1989; Brown & Bouton, 1993; Brown *et al.*, 1993; Oakley *et al.*, 2014). Furthermore, conflicting

topologies in molecular phylogenetic studies, which include C_3–C_4 intermediate species, indicate possible hybridisation events in the evolutionary history of these lineages (reviewed in Kadereit *et al.*, 2017). Hybridisation at some point in the history of a C_4 lineage might have perturbed the sequence of events depending on the photosynthetic phenotypes of the parental lineages. To investigate the possibility of a hybrid origin of a C_3–C_4 intermediate lineage, detailed phylogenomic studies are needed. For example, evidence of recent and ancient events of reticulate evolution in a phylogenomic study of *Flaveria* revealed a potential major role of hybridisation in the evolution of C_4 photosynthesis in that genus (Morales-Briones & Kadereit, 2022).

Here we conducted a study of a C_2 species aggregate at the population level and looked at C_4-adaptive traits to search for empirical evidence for the expected evolutionary shifts towards more C_4-like traits under stressful conditions according to the model proposed by Bräutigam & Gowik (2016). At the same time, we investigated the origin of this aggregate using a phylogenomic approach. The C_2 species we chose for our study were *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 (from this point forwards called *S. divaricata* agg.). The distribution area of the *S. 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.*, 2003). All four species of the *S. divaricata* agg. are salt tolerant, perennial shrubs with terete succulent leaves that are morphologically difficult to distinguish (Padrón Mederos, 2012) and seem mainly geographically defined. They all have a Kranz-like salsoloid leaf anatomy (Voznesenskaya *et al.*, 2013; Schüssler *et al.*, 2017). *Salsola divaricata* was categorised as a C_2 type I that is characterised by the photorespiratory CO_2 pump through GDC being confined to the bundle sheath cells while having C_3-like activity of C_4 enzymes (e.g. PEPC and NADP-ME). Their CO_2 compensation points *c.* 30 μmol mol^{-1} are intermediate to C_3 and C_4 species (Schüssler *et al.*, 2017). The *Salsola divaricata* agg. belongs to Salsoleae, a tribe rich in C_4 species, but also with several C_3 species and several C_3–C_4 intermediate species. The *Salsola divaricata* agg. forms a monophyletic, well supported subclade nested in a clade of C_3–C_4 intermediate and C_3 species with uncertain placement (Schüssler *et al.*, 2017).

The two aims of this study were to test whether the lineage is stable in its C_2 state or is shifting towards stronger C_4 physiology under more stressful climatic conditions on the eastern Canary Island and Morocco, and to test for a hybrid origin of the *Salsola divaricata* agg. To achieve these aims we studied C_4 adaptive traits at the population level in the 26 populations of the *S. divaricata* agg. distributed from La Gomera in the west to Morocco in the east and searched for more C_4-like phenotypes in the more arid eastern parts of the distribution area. We also analysed 991 loci from a transcriptome data set comprising C_3, C_4 and C_2 species of the Salsoleae and explored incongruences to determine if a hybridisation event is plausible in the lineage of the *S. divaricata* agg.

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 glasshouse at the Botanical Garden Mainz with multiple individuals per population. Most individuals of a population shared a mother plant, when this was not the case different mother plants are indicated with roman numbers in the individual names (LS No.). Plants were grown in custom mixed soil in standardised clay pots. Here, 14 h : 10 h, day : night cycles were artificially maintained with natural light and supplementary light providing a light intensity of 200–400 μmol m^{-2} s^{-1}. The minimum temperature at night was 18°C and daytime temperatures ranged between 25–35°C in summer and 20–25°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 once a week and every 2 wk with 2% saltwater (20 g NaCl per litre; 34.3 mM) from June 2015 to April 2021. Individuals in the dry treatment were only watered every 2 wk from June 2015 to March 2017. The analyses of the photosynthesis traits began in 2016. Table S1 lists the populations included in this study as well as the collection and voucher information.

Carbon isotope measurements

In C_3 plants, RuBisCo discriminates against assimilating atmospheric $^{13}CO_2$. C_4 plants exhibit much lower discrimination against $^{13}CO_2$ through its conversion to bicarbonate and assimilation by PEPC. Therefore, calculation of $\delta^{13}C$ values can be used to detect C_4 cycle activity in which more negative values indicate higher discrimination against fixing $^{13}CO_2$ (values for C_3 plants are −22 to −32° % and C_4 plants −9 to −16° %, Sage, 2016). We measured samples of dried leaves collected in the field from the same mother plants from which seeds were collected. We compared their $\delta^{13}C$ values with samples taken from the glasshouse cultivated offspring. We also determined and compared $\delta^{13}C$ values between and within glasshouse cultivated populations of *S. divaricata*. To determine carbon isotope composition, a standard procedure with Pee Dee Belemnite (PDB) limestone as the carbon isotope standard was used (Bender *et al.*, 1973). Leaf samples were dried in silica gel, and then 1–2 mg was placed in a tin capsule and combusted in an EuroVector elemental analyser (EuroVector, Pavia, Italy). After separating the resulting N_2 and CO_2 gases using gas chromatography, they were fed into the IsoPrime™ isotope ratio mass spectrometer (IRMS; GV Instruments Ltd (Micromass Ltd, Wilmslow, UK)) for determination of $^{13}C:{}^{12}C$ ratios (R). $\delta^{13}C$ values were determined according to $\delta^{13}C$ (‰) = 1000 $(R_{sample}/R_{standard})^{-1}$, where R is $^{13}C:{}^{12}C$. Measurements were taken at

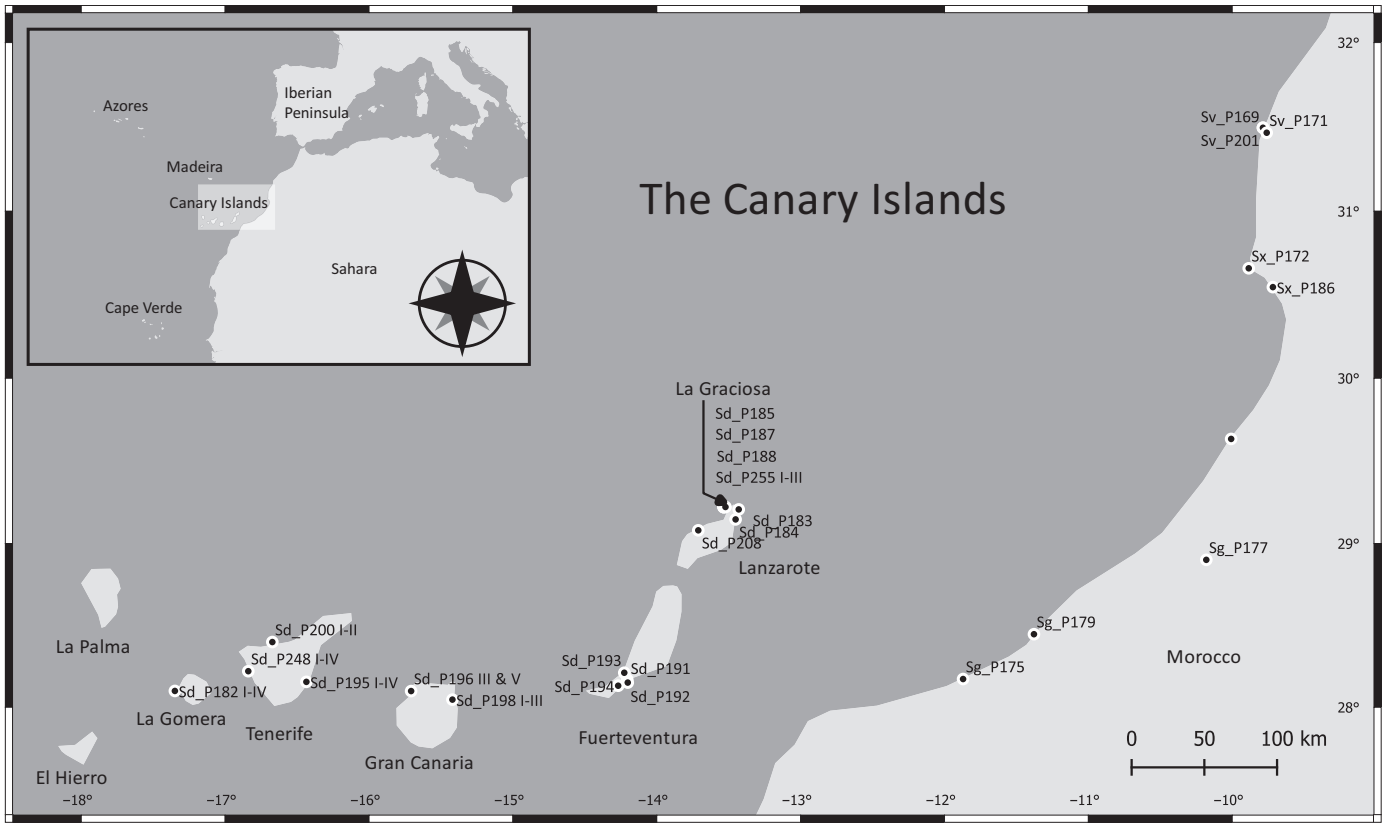

Fig. 1 Map of the collection locations of seeds and vouchers for the *Salsola divaricata* agg. (please refer to also Supporting Information Table S1 for voucher information). In total we analysed 26 populations of the *S. divaricata* agg. on the Canary Islands and Morocco. Species name abbreviation and 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 & Ibn Tattou, 1998) consider *S. verticillata* and *S. deschaseauxiana* as conspecific, while *S. gymnomaschala* seems more like *S. divaricata* s.s.

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CO_2 compensation point measurements

The CO_2 compensation point (Γ), the concentration at which there is no net uptake of CO_2 in the light, is a trait that is often used to identify C_4 species due to their relatively low values (usually less than 4 μmol CO_2 mol^{-1}) compared with C_3 species. We measured individuals of several populations per island of all species in the *S. divaricata* agg. ($n = 53$; between one and three technical replicates for each individual). The C_3 *S. webbii* and C_4 *S. oppositifolia* were measured for comparison. CO_2 compensation points were determined using the portable gas exchange measurement system GFS-3000 (Heinz Walz GmbH, Effeltrich, Germany) with the standard measuring head 3010-S equipped with a standard leaf area cuvette and an LED array (for details of the cuvette settings please refer to Methods S1). As a general calculation parameter in GFS-Win, we used leaf area that was measured using Easy Leaf Area v.1.02 or ImageJ 1.49d (Wayne Rasband, National Institutes of Health, USA) followed by a calculation according to the approximately cylindrical leaf shape. To determine the CO_2 compensation point, net rates of CO_2 assimilation were plotted against intercellular CO_2 concentration.

Phosphoenolpyruvate carboxylase activity measurements

Phosphoenolpyruvate carboxylase (PEPC) is a key enzyme in the C_4 pathway. It fixes atmospheric CO_2 after its conversion to HCO_3^- by carbonic anhydrase into the four-carbon compound that gave this photosynthesis pathway its name (Reyna-Llorens & Hibberd, 2017). C_4 plants show high levels of PEPC activity compared with C_3 plants and an increased activity is seen as an important stage in evolving from an intermediate to the full C_4 photosynthesis pathway (Sage *et al.*, 2012). PEPC activity measurements were conducted using the Tecan infinite M1000 reader (Tecan Trading AG, Männedorf, Switzerland) and a Greiner UV-Star 96-well plate (Sigma-Aldrich Chemie GmbH, Munich, Germany). For details of sample preparation please refer to Methods S1.

The assay for 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 based on the linear part of the progress curve where a = SU (μmol ml^{-1} min^{-1}) V_{assay} (ml)/(1000 V_{enzyme} (ml) c_{Chla+b} (mg ml^{-1})), where SU is the substrate turnover rate, 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 v.3.1.2 (R Core Team, 2014).

Transcriptome processing and nuclear phylogenetic analyses

To evaluate the potential hybrid origin of the *S. divaricata* agg., we assembled a dataset comprising 13 transcriptomes representing 12 C_3, C_4 and C_2 species of Salsoleae. The data set included 10 publicly available transcriptomes and three newly sequenced species on an Illumina HiSeq 2500 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 three closely related Amaranthaceae species as outgroups. Table 1 lists all samples with their photosynthesis type, ploidy if known and sequence read archive (SRA) accession number.

Raw read processing, transcriptome assembly, low-quality and chimeric transcript removal, transcript clustering into putative genes, translation, final coding sequences (CDS) redundancy assessment, and homology and orthology inference were carried out following Morales-Briones *et al.* (2021; for details please refer to Methods S1). Briefly, an all-by-all BLASTN search was performed, and putative homologues groups were clustered using MCL (van Dongen, 2000). Homologue trees were built using RAxML (Stamatakis, 2014) and spurious tips were removed with TreeShrink (Mai & Mirarab, 2018). Monophyletic and paraphyletic tips that belonged to the same taxon were removed, leaving one tip with the most aligned characters to obtain final homologues. Orthology inference was carried out following the 'monophyletic outgroup' approach from Yang & Smith (2014), keeping only orthologue groups with all 17 taxa present.

We applied concatenation and coalescent-based methods for phylogenetic reconstruction. For the concatenation approach, we prepared a supermatrix by keeping only orthologue alignments with at least 300 bp. 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 multispecies 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 concordant and conflicting bipartitions on each node of the species trees. We calculated both the ICA scores and conflict analyses using 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 1000 replicates (for details please refer to Methods S1).

Assessment of hybridisation

To detect possible hybridisation, 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 hybridisations and test whether the species network fitted our gene trees better than a 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 please refer to Methods S1).

Plastome assembly and phylogenetic analysis

To investigate phylogenetic signals from plastid sequences, *de novo* assemblies were carried out with the FAST-PLAST v.1.2.6

Table 1 List of the 17 samples in the transcriptome phylogeny; with photosynthesis type, ploidy (Kew C value database 2018; NA, not available) and NCBI sequence read archive (SRA) accession number.

Species	Tribe	Ploidy	Photosynthesis type	SRA accession no.
Anabasis articulata Choul. ex Pomel	Salsoleae	NA	C_4	SRR6435311
Halogeton glomeratus (M.Bieb.) C.A.Mey.	Salsoleae	2×	C_4	SRR1503502
Haloxylon ammodendron (C.A.Mey.) Bunge	Salsoleae	2×	C_4 (C_3 cotyledon)	SRR1697346
Hammada scoparia (Pomel) Iljin	Salsoleae	2×	C_4 (C_3 cotyledon)	ERR2060287
Kali collina Akhani & Roalson	Salsoleae	2×	C_4	SRR6435349
Salsola divaricata Moq. (Pop.184)	Salsoleae	4×	C_2	ERR2060298
Salsola divaricata Moq. (Pop.198)	Salsoleae	4×	C_2	ERR2060294
Salsola genistoides Juss. ex Poir.	Salsoleae	4×	C_3	SRR14783909
Salsola montana Litv.	Salsoleae	NA	C_3 proto-kranz	SRR14783908
Salsola oppositifolia Desf.	Salsoleae	8×	C_4	ERR2060302
Salsola soda L.	Salsoleae	2×	C_4 (C_3 cotyledon)	SRR3544552
Salsola verticillata Schousb. (Pop.171)	Salsoleae	4×	C_2	SRR14783907
Salsola webbii Moq.	Salsoleae	4×	C_3	ERR2060308
Caroxylon vermiculatum (L.) Akhani & Roalson	Caroxyloneae	NA	C_4	SRR6435345
Bassia scoparia (L.) A.J.Scott	Camphorosmeae	2×	C_4	ERR364385
Eokochia saxicola (Guss.) Freitag & G.Kadereit	Camphorosmeae	NA	C_3	SRR6435348
Beta vulgaris subsp. *vulgaris* L.	Beteae	2×	C_3	SRX335625

pipeline (https://github.com/mrmckain/Fast-Plast) using the filtered organelle reads obtained from the transcriptome raw read processing. An 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 nonparametric BS replicates for branch support (for details please refer to Methods S1). Additionally, we used QS with 1000 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. showed a tendency to group with either a C_3 or a C_4 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 C_4-associated proteins (Lauterbach *et al.*, 2017b) as well as two C_4-associated transcription factors (Lauterbach *et al.*, 2017a). Gene annotation and pathway assignment can be found in the results (Fig. 7). We examined whether the *S. divaricata* agg. was sister to the C_3 species *S. montana* or the C_4 clade. When there were several gene copies or weak bootstrap support, we additionally noted whether species of the *S. divaricata* agg. were in a clade with C_3 or C_4 species (for details please refer to Methods S1).

Results

Carbon isotope measurements

Salsola divaricata plants growing in the wild on the Canary Islands generally had higher carbon isotope ($\delta^{13}C$) values (−21 to −28; mean −24.44, $n = 30$) than those grown from seeds under glasshouse conditions (−22 to −34; mean −27.77, $n = 165$). Overall, the $\delta^{13}C$ values of the *S. divaricata* agg. showed a distinct range but not a single individual (stressed or not stressed) came close to a C_4-like $\delta^{13}C$ value of more than −16 (Fig. 2). The control and drought treatment showed no significant difference for the $\delta^{13}C$ values among populations (Kruskal–Wallis χ^2 = 5.1081 and 8.61, df = 5, n = 165 and 36, P-value = 0.40 and 0.13, respectively). In the salt treatment there was a significant difference in the $\delta^{13}C$ values among populations (Kruskal–Wallis χ^2 = 15.47, df = 5, n = 36, P-value = 0.009), which was due to significantly higher $\delta^{13}C$ values in salt-treated individuals of populations, 191 compared with 182 and 194. We tested for differences in the carbon isotope values between treatments within populations of the *Salsola divaricata* agg. and found a significant difference for populations 182, 191 and 198 (Fig. 3). However, there was no significant difference among the three treatments for populations 184, 188 and 194 (Fig. 3). After grouping $\delta^{13}C$ 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, Tenerife and the mainland, respectively. All other differences were not statistically significant. We found that the populations of *S. verticillata*, *S. deschaseauxiana* and *S. gymnomaschala*

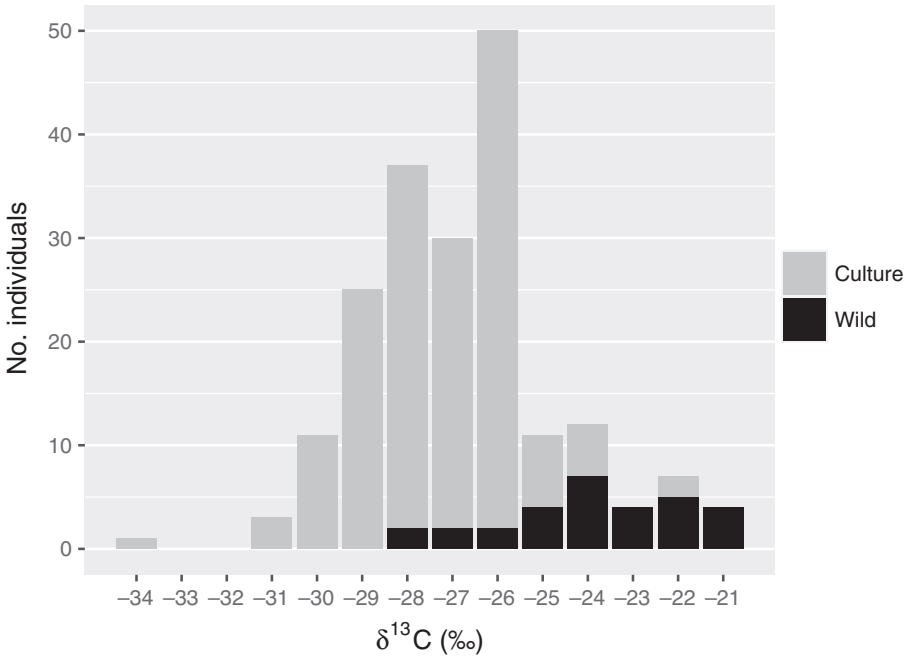

Fig. 2 Distribution of $\delta^{13}C$ (‰) of 195 samples of the *Salsola divaricata* agg. Of these, 30 were collected from plants growing in the wild and 165 from plants in cultivation at the Botanical Garden Mainz. This includes 36 individuals in dry treatment and 36 in salt treatment; all others are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

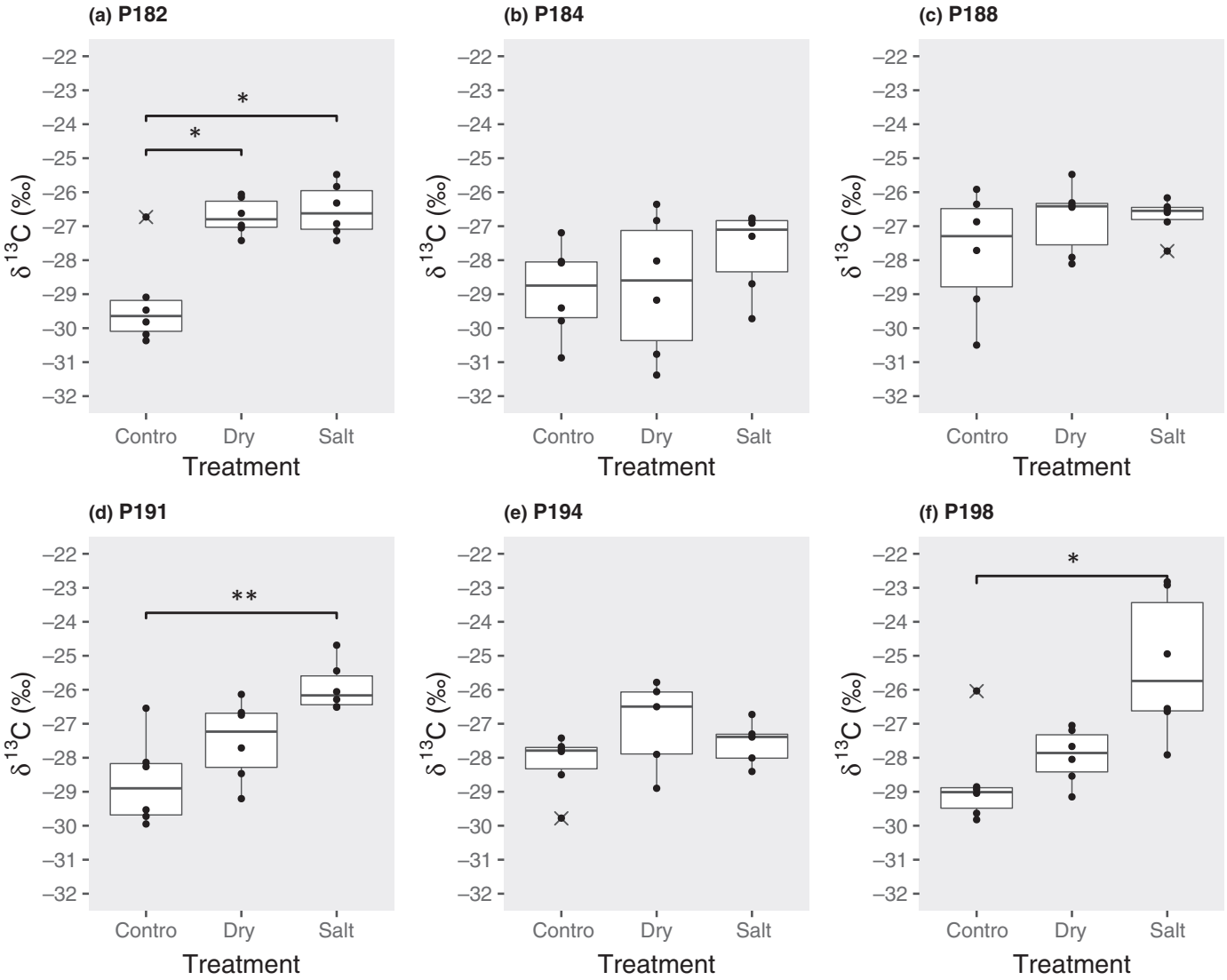

Fig. 3 Comparison of carbon isotope values ($\delta^{13}C$ (‰)) between treatments within populations (P) of the *Salsola divaricata* agg. Significant differences were found for 182 populations from La Gomera (a; Kruskal–Wallis $\chi^2 = 7.94$, $df = 2$, $n = 18$, P-value = 0.02), 191 from Fuerteventura (d; Kruskal–Wallis $\chi^2 = 10.84$, $df = 2$, $n = 18$, P-value = 0.004), and 198 from Gran Canaria (f; Kruskal–Wallis $\chi^2 = 8.84$, $df = 2$, $n = 18$, P-value = 0.01). No significant differences were found between the three treatments for 184 from Lanzarote (b; Kruskal–Wallis $\chi^2 = 2.33$, $df = 2$, $n = 18$, P-value = 0.31), 188 from La Graciosa (c; Kruskal–Wallis $\chi^2 = 0.78$, $df = 2$, $n = 18$, P-value = 0.68), and 194 from Fuerteventura (e; Kruskal–Wallis $\chi^2 = 2.27$, $df = 2$, $n = 18$, P-value = 0.32). Asterisks indicate significant differences: *, $P < 0.05$; **, $P < 0.01$. Circles indicate individual data points, crosses are outliers, horizontal lines indicate the median and first and third quartiles, and the whiskers connect the minimum and maximum data points excluding outliers.

on the Moroccan mainland often had higher $\delta^{13}C$ values than the populations of *S. divaricata* on the Canary Islands (the nonstressed control groups that are regularly watered; Fig. S1). However, due to very different sample sizes further statistical tests including all populations were inconclusive.

Overall, these results showed variation in $\delta^{13}C$ values among populations associated with source population and growth conditions; but all were well within the range typical of C2 and C_3 species. The significantly higher $\delta^{13}C$ values of salt-treated individuals indicated that salinity rather than drought affects the $\delta^{13}C$ in *Salsola divaricata* agg. and could be responsible for the occasional observation of relatively high $\delta^{13}C$ values of our samples collected in the wild close to the shoreline.

CO_2 compensation point measurements

The CO_2 compensation points (Γ) of the four species of the *Salsola divaricata* agg. lay between those of the C_3 (*Salsola webbii*) and C_4 species (*Salsola oppositifolia*) as expected (between 20 and 30 µmol CO_2 mol^{-1}; Fig. 4a). The mean values of *S. deschaseauxiana* and *S. verticillata* were slightly lower than those of *S. divaricata* and *S. gymnomaschala*. When looking at individual measurements we again observed a broad range from 17 to 40 µmol CO_2 mol^{-1} with one outlier at 9 µmol CO_2 mol^{-1} (Fig. S2). Populations growing on the more arid islands such as Lanzarote and Fuerteventura or in Morocco did not show lower CO_2 compensation points. Due to varying values within

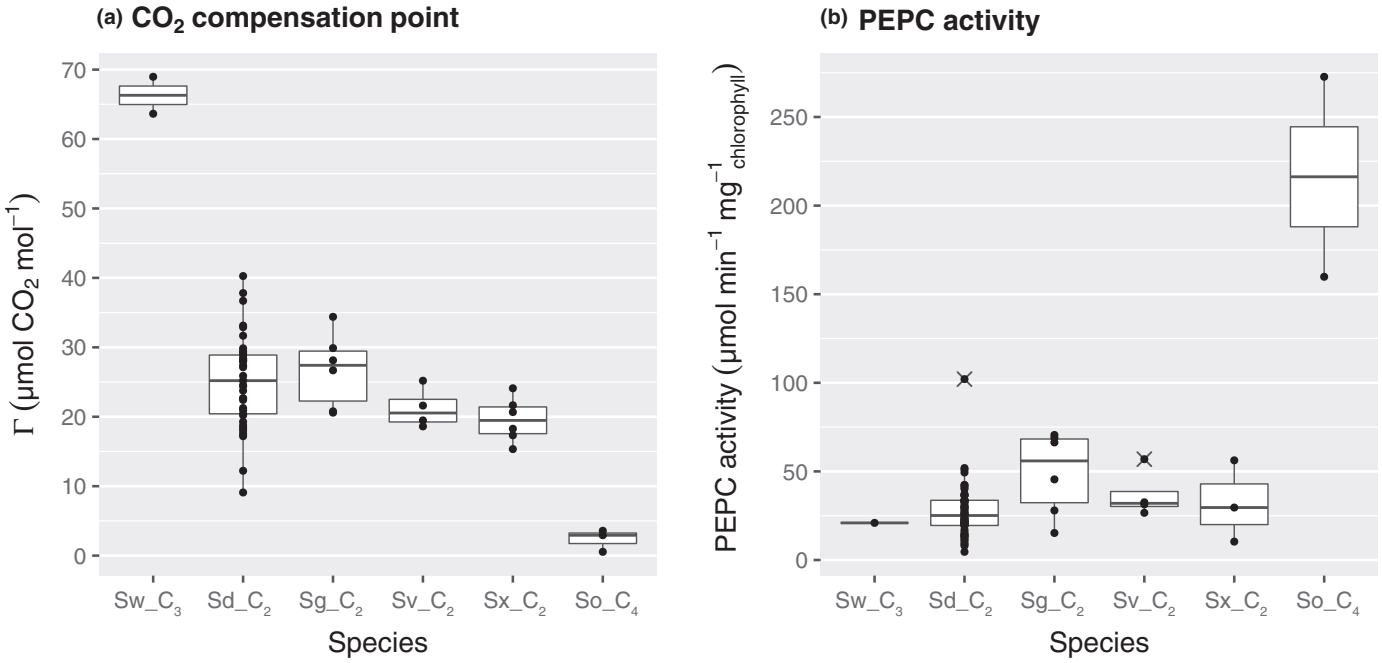

Fig. 4 Comparison of CO_2 compensation points and PEPC activity. (a) CO_2 compensation points (Γ in µmol CO_2 mol^{-1}) of the separate species of the C_2 *Salsola divaricata* agg. (Sd_C_2 = *Salsola divaricata*, mean 25.14 µmol CO_2 mol^{-1}, $n = 21$; Sg_C_2 = *S. gymnomaschala*, mean 26.75 µmol CO_2 mol^{-1}, $n = 4$; Sv_C_2 = *S. verticillata*, mean 22.21 µmol CO_2 mol^{-1}, $n = 4$; Sx_C_2 = *S. deschaseauxiana*, mean 19.56 µmol CO_2 mol^{-1}, $n = 4$) compared with the values of C_3 species *Salsola webbii* (Sw_C_3, 66.3 µmol CO_2 mol^{-1}, $n = 2$) and C_4 species *Salsola oppositifolia* (So_C_4, 2.35 µmol CO_2 mol^{-1}, $n = 3$). We measured between one and three technical replicates per individual. (b) PEPC activity (µmol min^{-1} $mg_{chlorophyll}{}^{-1}$) of the separate species of the C_2 *Salsola divaricata* agg. (*Salsola divaricata*, mean activity 33 µmol min^{-1} $mg_{Chlorophyll}{}^{-1}$, $n = 82$; *S. gymnomaschala*, mean 46.8 µmol min^{-1} $mg_{Chlorophyll}{}^{-1}$, $n = 10$; *S. verticillata*, mean 37.4 µmol min^{-1} $mg_{Chlorophyll}{}^{-1}$, $n = 7$; *S. deschaseauxiana*, mean 27.6 µmol min^{-1} $mg_{Chlorophyll}{}^{-1}$, $n = 6$) compared with C_3 *S. webbii* (mean 30 µmol min^{-1} $mg_{Chlorophyll}{}^{-1}$, $n = 1$) and C_4 *S. oppositifolia* (mean 216 µmol min^{-1} $mg_{Chlorophyll}{}^{-1}$, $n = 2$). Circles indicate individual data points, crosses are outliers, horizontal lines indicate the median and first and third quartiles, and the whiskers connect the minimum and maximum data points excluding outliers.

each population (Fig. S2) and the relatively small sample sizes (one to two individuals per population) we did not conduct further statistical analysis.

Phosphoenolpyruvate carboxylase activity measurements

First, we compared the PEPC activity of the separate species of the *Salsola divaricata* agg. to the values of the closely related C_3 species *Salsola webbii* and C_4 species *Salsola oppositifolia*. C_3 and C_4 values were as expected clearly distinguishable (Fig. 4b). The values of the C_2 species were closer to those of the C_3 species and much lower than the C_4 species (Fig. 4b). We used a Kruskal–Wallis test to determine if statistically significant differences existed between C_2 species (Kruskal–Wallis $\chi^2 = 9.94$, $df = 3$, 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 µmol min^{-1} $mg_{Chlorophyll}{}^{-1}$ and *S. gymnomaschala* in Morocco having a median of 48.32 µmol min^{-1} $mg_{Chlorophyll}{}^{-1}$. Due to the high level of variation within species we took a closer look at 14 populations of *S. divaricata* ($n = 5$–7 per population) and observed a significant difference between them (Kruskal–Wallis $\chi^2 = 34.64$, $df = 13$, P-value = 0.001; please refer to also Fig. 5a). When grouped according to island, we found a significant difference (Kruskal–Wallis $\chi^2 = 22.199$, $df = 5$, P-value = 0.0005) which was 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 glasshouse in *S. divaricata* (Kruskal–Wallis $\chi^2 = 1.21$, $df = 2$, P-value = 0.55). Again, we observe a high variation of PEPC activity values within populations and islands.

Linear regression to test for correlation between PEPC activity and carbon isotope ($\delta^{13}C$) values (multiple $R^2 = 0.008$, F-statistic = 0.35, $df = 44$, P-value = 0.56), between PEPC activity and the CO_2 compensation point (Γ) (multiple $R^2 = 0.04$, F-statistic = 1.19, $df = 29$, P-value = 0.28), and between $\delta^{13}C$ values and Γ (multiple $R^2 = 0.017$, F-statistic = 0.46, $df = 27$, P-value = 0.5) recovered no significant correlation among the three.

Phylogenomic analyses

The raw reads for the newly generated transcriptomes are available from the NCBI SRA (Table 1). The 991 final orthologues had alignments of lengths between 315 and 6621 bp. The concatenated matrix comprised 1427 449 aligned columns with overall matrix occupancy of 86% (please refer to Table S2 per details).

The ASTRAL species tree and the concatenated RAxML tree (Fig. S3) had similar topologies with most nodes receiving the

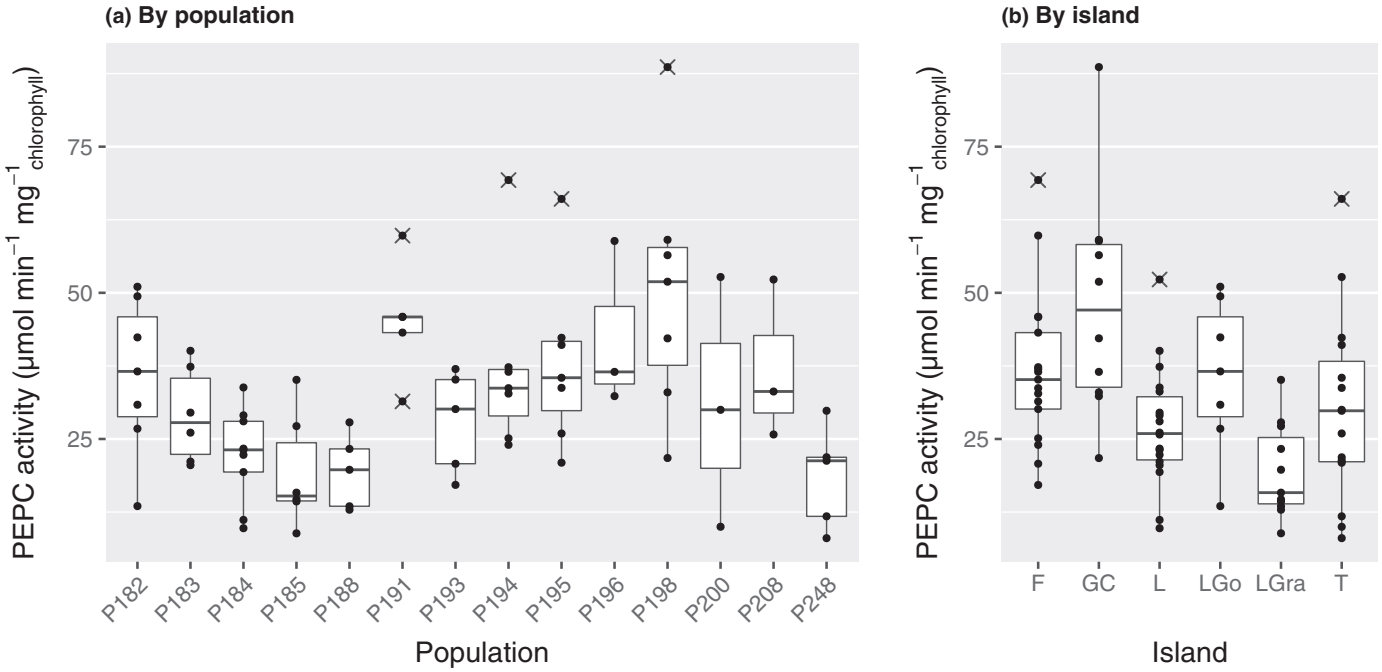

Fig. 5 Boxplots of PEPC activity (µmol min^{-1} $mg_{chlorophyll}{}^{-1}$) of *Salsola divaricata* Moq. (a) Values grouped by population (P). (b) Values grouped by island; F, Fuerteventura; GC, Gran Canaria; L, Lanzarote; LGo, La Gomera; LGra, La Graciosa; T, Tenerife. Circles indicate individual data points, crosses are outliers, horizontal lines indicate the median and first and third quartiles, and the whiskers connects the minimum and maximum data points excluding outliers.

maximum support (BS = 100%; LPP = 1). The only difference between the two topologies was 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 (Figs S3, S4) showed high support for the monophyly of Salsoleae and the *S. divaricata* agg., while signalling conflict for the remaining taxa. The placement of *S. genistoides* had low support with only 261 (of 794) concordant gene trees (ICA = −0.45) and a QS score (0.22/0/0.98) suggesting an alternative topology. Monophyly of the *S. divaricata* agg. clade composed of *S. divaricata* + *S. verticillata* had strong support with 808 (of 913) concordant gene trees (ICA = 0.71) and maximum QS support (1/–/1; i.e. all sampled quartets supported that branch). However, the two samples of *S. divaricata* showed signals of alternative topologies with only 332 (of 900) gene trees supporting their sister relationship (ICA = 0.56) and QS counter-support (−0.25/0/1). The sister relationship of *S. divaricata* agg. and the C4 remaining Salsoleae had low support with only 178 (of 705) concordant gene trees (ICA = 0.29) and low QS support (0.085/0/1 (ASTRAL); (0.11/0/1) (RAxML)) suggesting an alternative topology. The placement of the C4 *Anabasis articulata* and *S. 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 *S. oppositifolia*, *Halogeton*, *Hammada* and *Haloxylon* was supported only by 280 (of 692L ICA = 0.24), but had maximum QS support while, within the clade, all relationships had low gene tree and QS support signalling 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; Figs 6, S5). *Caroxylon vermiculatum*, *Kali collina* and *S. genistoides* form a well supported clade (BS = 100; QS = 0.56/0/1) that is sister to all Salsoleae. *S. webbii* and *Anabasis articulata* form a grade with strong support (BS = 100; QS = 0.6/0/0.99 (*S. webii*); 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 *S. verticillata* + *S. montana* (BS = 100; QS = 0.61/0/1) and the two samples of *S. 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 *S. oppositifolia*, *Halogeton*, *Hammada* and *Haloxylon*.

Assessment of hybridisation

Model selection preferred a network as a better model than any bifurcating tree (Table 2). A network with three reticulation events was the best model overall (PHYLONET-4-hybridisation search; AIC_c = 37 875.93; BIC = 38 054.302; Fig. 6c). The next model was a significantly worse model (also a network with three reticulation events (PHYLONET-3-hybridisation search) with ΔAIC_c and ΔBIC of 753.208. The *S. divaricata* agg. clade was shown as the product of a hybridisation event between *S. soda* (C_4) (inheritance probability P_i = 0.346) and *S. montana* (C_3) (P_i = 0.654). Then the C_4 clade *S. oppositifolia* + *Halogeton* + *Hammada* + *Haloxylon* was the product of reticulation between *S. soda* (C_4) (P_i = 0.846) and the stem lineage of the C_2 *S. divaricata* agg. (P_i = 0.154). These two clades were also recovered as

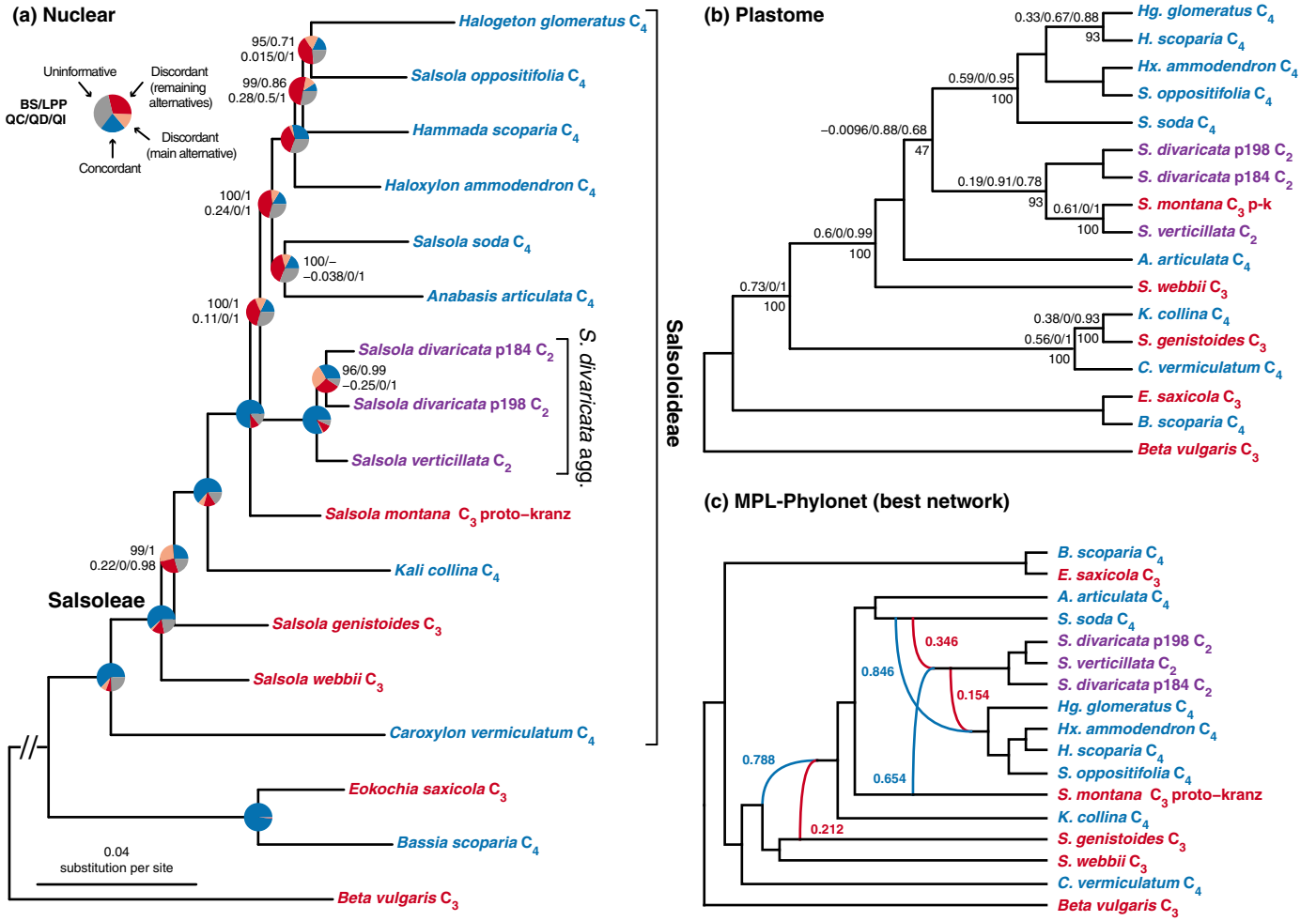

Fig. 6 (a) Maximum likelihood phylogeny of Salsoloideae inferred from the RAxML analysis of the concatenated 991-nuclear gene supermatrix from the 'monophyletic outgroup' (MO) orthologues. Bootstrap support and local posterior probabilities (BS/LPP) are shown above branches. Nodes with full support (BS = 100/LPP = 1) are not shown. Em dashes (—) denote alternative topology compared with the ASTRAL tree (Supporting Information Fig. S3). Quartet sampling (QS) scores are shown below branches. QS score: Quartet concordance (QC)/Quartet differential (QC)/Quartet informativeness (QI). Full QS support (1/–/1) not shown. Pie charts represent the proportion of gene trees that supports that clade (blue), the proportion that supports the main alternative bifurcation (peach), the proportion that supports the remaining alternatives (red), and the proportion (conflict or support) that have < 50% bootstrap support (grey). 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. Please refer to 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.

hybrids in the networks with one, two and three reticulation events with similar parental lineages and inheritance probabilities (Fig. S6). The third event was a deeper reticulation within Salsoleae that showed that most species of the group (excluding *Caroxylon*) were a product of an ancient hybridisation event between *S. genistoides* (P_i = 0.212) and the sister lineage of *S. genistoides* + *S. webbii* (P_i = 0.788). The HYDE analysis resulted in 1680 hybridisation tests, of which 298 triples were significant (Table S3; Fig. S7). The significant triples showed that 12 out of 13 individuals of Salsoleae are involved in multiple hybridisation events, which is mostly in agreement with the nested reticulation events detected with PHYLONET. All sampled accessions of the *S. divaricata* agg. showed similar hybridisation patterns (Fig. S7; that is same potential parental lineages and admixture parameter (γ)), consistent with a single ancient hybrid origin of *S. divaricata* agg. as shown in the PHYLONET analyses. Overall, the admixture parameter (γ) ranged from 0.013 to 0.986 (average 0.458). These results indicated multiple, complex hybridisations within Salsoleae. Additionally, HYDE also identified *Eokochia saxicola* as a hybrid (Table S3; Fig. S7).

Analysis of individual genes involved in photosynthesis or photorespiration

The 50 genes ranged from 7 to 65 sequences due to variation in the number of accessions and gene copies (Table S4). Summarising all genes, *Salsola divaricata* agg. appeared 22 times as sister to C_3 *S. montana* and 26 times as sister to C_4 species (Fig. 7;

Table 2 Model selection between bifurcating trees and networks.

Tree/network	$\text{Log}_e L$ (log likelihood)	k	h[1]	AIC_c	ΔAIC_c	BIC	ΔBIC
Astral-nuclear	−19 754.54543	31	N/A	39 573.130	1697.190	39 722.951	1668.649
RAxML-nuclear	−19 741.89558	31	N/A	39 547.830	1671.891	39 697.651	1643.349
IQ-Tree-plastome	−22 836.08408	31	N/A	45 736.207	7860.268	45 886.028	7831.726
PhyloNet. 1-hybridisation search (network 1)	−19 451.79848	31	1	38 967.636	1091.696	39 117.457	1063.155
PhyloNet. 1-hybridisation search (network 2)	−19 605.00833	33	1	39 278.323	1402.383	39 437.674	1383.372
PhyloNet. 1-hybridisation search (network 3)	−19 624.56237	33	1	39 317.431	1441.491	39 476.782	1422.480
PhyloNet. 2-hybridisation search	−19 435.04197	35	2	38 942.674	1066.734	39 111.539	1057.237
PhyloNet. 3-hybridisation search	−19 276.12872	37	3	38 629.147	753.208	38 807.510	753.208
PhyloNet. 4-hybridisation search	−18 899.52481	37	3	37 875.940	0.000	38 054.302	0.000
PhyloNet. 5-hybridisation search	−19 283.48635	41	5	38 652.512	38 652.512	38 849.820	38 849.820

The best model (lowest score) is the network with three hybridisation events from PhyloNet (best MPL run allowing four hybridisation events). k = no. branch lengths + hybridisation probabilities [$(2n - 3) + (2h)$]; h = number of hybridisation events; N/A, not applicable. All trees and networks had 17 taxa (n). Networks and bifurcating trees log likelihood values were inferred with 991 loci.
[1]The number of reticulation events in the best networks of a search is not necessarily the same number of reticulation events allowed in the search.

Table S4). *Salsola divaricata* agg. was not detected in three genes (Isocitrate lyase, RuBisCo subunits). 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 C_3 and/or C_4 species. In these 23 gene trees, sequences of the *S. divaricata* agg. appeared 21 times with C_3 and 26 times with C_4 species. Summarising the results for genes typical for C_4 pathways, the *S. divaricata* agg. was 27 times sister to and/or nested in a C_3 clade and 32 times sister to and/or nested in a C_4 clade). Taking into consideration bootstrap support (BS ≥ 70), the numbers reduced to 18 times sister to and/or nested in a C_3 clade and 23 times sister to and/or nested in a C_4 clade for C_4 genes. For the two C_4-associated transcription factors, *S. divaricata* agg. was sister to the C_4 clade for SHORTROOT with strong support (BS = 86). The sister relationship to the C_4 clade was moderately supported for BEL1-like homeodomain 7 (BS = 57 in clade with C_4). For the genes assigned to the glyoxylate cycle, *S. divaricata* agg. had multiple copies that were grouped with either C_3 or C_4 with high support (BS > 80). The gene for isocitrate lyase was missing in the *S. divaricata* agg. and *S. montana*. *Salsola divaricata* agg. is also missing in the alignments for the RuBisCo subunits. For photorespiration-associated genes, many species, including those of *S. divaricata* agg., had multiple copies, even though during the transcriptome processing putative genes were inferred to remove isoforms and assembly artefacts. Copies of *S. divaricata* agg. were nested within C_3 clades with strong support (nine times, BS > 70). Affinity to the C_4 clade was well supported in 13 of 19 cases, in which they appeared as sister or nested within the clade. Just like the *S. divaricata* agg., the C_4 clade was also not monophyletic in most cases. Clade assignment for all 50 genes is listed in Table S4 and the individual trees are shown in the Fig. S8.

Discussion

Hybridisation experiments with parental lineages exhibiting different photosynthetic types have shown the transferability of C_4 traits (Apel *et al.*, 1988). For a few examples, it has been suggested that this might also occur in nature (Dunning *et al.*, 2017); that could mean an immediate adaptive advantage of the hybrid lineage in more stressful environments. A phylogenomic study of *Flaveria* revealed a major role of hybridisation and transgressive segregation in C_4 evolution (Morales-Briones & Kadereit, 2022). In this study we aimed to infer the phylogenetic origin of the C_3–C_4 intermediacy of the *Salsola divaricata* agg. and possible phenotypic variation across a steep climate gradient. *Salsola divaricata* is currently classified as a C_2 type I according to Sage *et al.* (2014). According to the model of C_4 evolution, an increase in the PEPC activity should be expected under more stressful (more carbon-deficient) conditions in the more arid areas (Lanzarote, Fuerteventura, Morocco) with a transition to the C_2 type II or even C_4-like intermediate type, resulting in a reduction in photorespiration (Sage *et al.*, 2014; Bräutigam & Gowik, 2016). Alternatively, C_2 photosynthesis in *Salsola divaricata* agg. may represent a stable condition across mesic and arid growing conditions, as presently observed in this study.

Carbon isotope measurements depict a stress reaction in the *S. divaricata* agg.; but without occurrence of a more C_4-like photosynthesis in populations growing in drier environments

Although samples collected in the wild had slightly higher $\delta^{13}C$ values than those sampled in our glasshouse (stressed or control), there is still a clear difference to typical values of C_4 species. None of the samples showed a $\delta^{13}C$ value typical for a C_4-like or a C_4 plant, which would be *c.* −9 to −16‰ (Sage, 2016); instead, all samples had values falling within the range typical for C_3 species, which would be *c.* −22 to −32‰ (Sage, 2016). In C_3 plants the $\delta^{13}C$ values of leaves depend on several factors including stomatal conductance and capacity to capture CO_2 in photosynthesis, which is dependent on light intensity, growth temperature, photochemistry and the capacity for carbon assimilation. Plants growing in the glasshouse 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

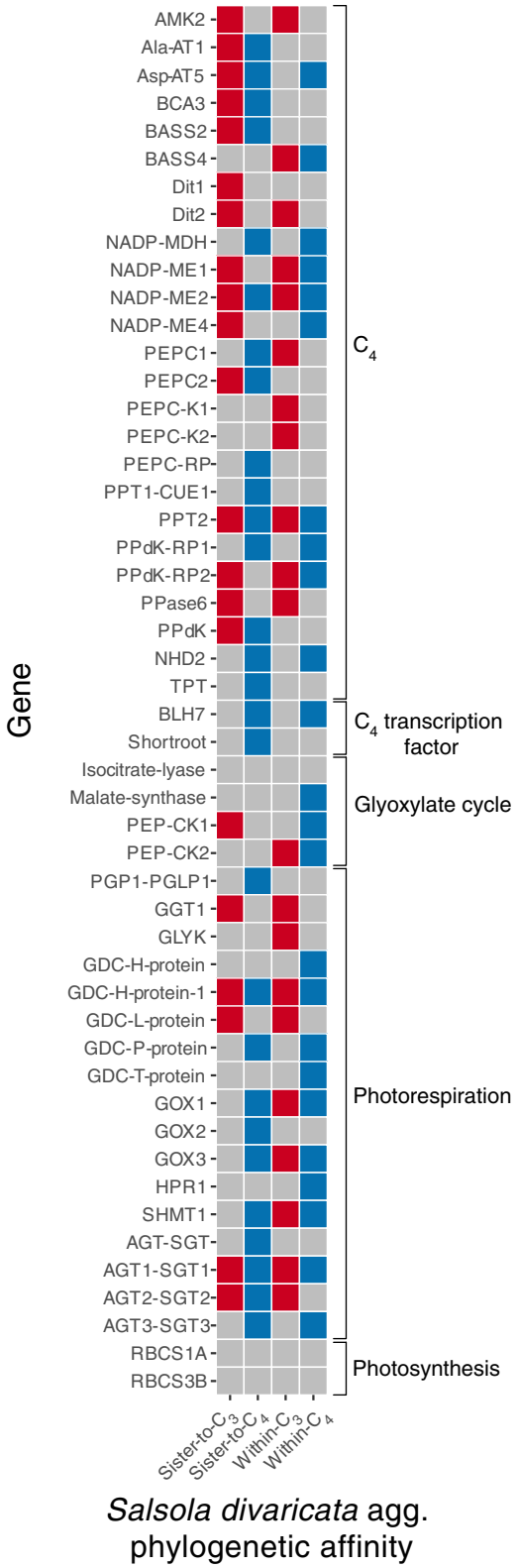

Fig. 7 Summary of the phylogenetic affinity of the *Salsola divaricata* agg. in analysed photosynthetic gene trees. Red and blue coloured boxes represent phylogenetic affinity with C_3 or C_4 species in the specified gene, respectively. Grey boxes denote no affinity. Gene with only grey boxes denotes that the gene was not detected in the *Salsola divaricata* agg. transcriptomes we sequenced. For detailed phylogenetic affinity please refer to Supporting Information Table S4; Fig. S8.

results suggested a greater stomatal limitation on photosynthesis in the field-grown plants, which resulted in a low intercellular CO_2 concentration. This will change the discrimination against the heavier C isotope, as RuBisCo will capture more $^{13}CO_2$, resulting in slightly higher $\delta^{13}C$ values (von Caemmerer, 1992).

We observed variation in the $\delta^{13}C$ values. Populations on the Moroccan mainland often have higher values than the populations on the Canary Islands; but there were many exceptions in the 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, the observed variation is likely to be due to plasticity or local, microclimatic differences. In any case, there were no indications of populations exhibiting C_4-like photosynthesis based on the $\delta^{13}C$ values.

Salsola divaricata plants from all populations grown for several years under salt stress in the glasshouse showed clear morphological differences in comparison with 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 $\delta^{13}C$ value. The other three populations from Lanzarote, La Graciosa and Fuerteventura had slightly, but not significantly higher, values for the salt treatment compared with the control group. This suggests that *Salsola divaricata* can adapt to salt stress by increasing water storage and decreasing stomatal conductance and it explains some of the $\delta^{13}C$ value variation found in the wild, as salinity certainly varies among sites.

C_3–C_4 intermediate CO_2 compensation points for *S. divaricata* agg. support a stable C_2-state

The CO_2 compensation points of the *S. divaricata* agg. varied, but they were well within the range of other C_2 species (Sage *et al.*, 2014 and reference therein) and they were significantly lower than the values of the C_3 species *S. webbii* and significantly higher than those of the C_4 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 showed somewhat lower values, many individuals from similar climatic environments (e.g. *S. gymnomaschala* or individuals of *S. divaricata* from Fuerteventura or Lanzarote) showed higher values, which skewed a possible relationship of climate and lower Γ values. Sage *et al.* (2014) suggested that a Γ value below 10 μmol CO_2 mol^{-1} characterises the transition from C_2 Type I to C_2 type II and represents an evolutionary progression towards C4. An accessory C4 metabolic cycle is present in C_2 type II plants and was so far only observed in several species of *Flaveria* and *Mollugo verticillata* (Edwards & Ku, 1987; Sage *et al.*, 2018). None of the Γ measurements of 53 individuals of the *Salsola divaricata* agg. showed an indication of an accessory C4 metabolic cycle and a significantly higher

carboxylation using PEPC, therefore indicating a stable C_2-state for *S. divaricata* agg.

So far, the analysed traits seem to be independent between populations and islands and showed substantial variation. On Lanzarote, for example, populations of *S. divaricata* had comparably high Γ values and medium-to-low carbon isotope values compared with other populations.

PEPC activity has little or no correlation with Γ values or carbon isotope values in *S. divaricata* agg.

The PEPC activity of the *S. divaricata* agg. was similar to the C_3 species *S. webbii*. Even though there was a high standard deviation within C_4 *S. oppositifolia*, none of the C_2 individuals was even close to a C_4-like value. The activity of PEPC for *S. verticillata* and *S. deschaseauxiana* was slightly lower than those for *S. divaricata*, therefore their slightly lower Γ values and slightly higher carbon isotope values were not due to increased CO_2 fixation by an optimised PEPC. Therefore, *S. verticillata* and *S. deschaseauxiana* did not seem to be closer to a more C_4-like state than *S. divaricata* or *S. gymnomaschala*.

The PEPC activity in the *S. divaricata* agg. is highly variable and showed only a weak correlation with carbon isotope values and CO_2 compensation points, as discussed above for *S. verticillata*. We would expect a higher PEPC activity in the salt treatment or the more arid locations, for example Lanzarote or Fuerteventura, however that was not the case. Although there were statistically significant differences between islands, it was not possible to predict a value for PEPC activity given the location as there was 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 glasshouse. We could only detect a very weak correlation between PEPC activity, CO_2 compensation points and carbon isotope values, supporting a rather independent distribution of the traits and their high phenotypic variation.

The independent variation in three photosynthetic traits (carbon isotope value, CO_2 compensation point and PEPC activity) does not support a directed selection towards C_2 type II or C_4-like photosynthesis in any of the populations studied, not even those growing in semidesert conditions. By contrast, our trait observations are more in line with the results of hybridisation experiments. Summarising the physiology of advanced generations of interspecific C_3 and C_4 hybrids, Brown & Bouton (1993) stated that the 'correlation among photosynthetic traits was low' and that indicates 'a high degree of independence, both genetic and physiological, among C_4 traits'. As found in our study, the hybridisation experiment of C_3 and C_4 *Atriplex* (Oakley *et al.*, 2014) showed a distinct range of Γ values (in nine F_2 individuals from 25–45 μmol CO_2 mol^{-1} and one C_4-like value). Brown & Bouton (1993) hypothesised that some of the photosynthetic traits measured could be more C_4-like in a still functioning C_3 metabolism, such as Γ, while others just mirrored the physiological C_3 default condition, such as enzyme activity. This, together with the phylogenomic results and photosynthetic gene tree analyses (please refer to later), supported the hypothesis of a hybrid origin of *Salsola divaricata* agg. and subsequent stabilising selection forming an evolutionary stable C_2 lineage.

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

The Salsoleae species tree showed a high level of conflict including *S. divaricata* agg, despite high BS. The assessment of hybridisation events using PhyloNet and HyDe showed multiple hybridisation events within Salsoleae in which the *Salsola divaricata* agg. was likely to be involved. The incomplete sampling of Salsoleae did not allow us to identify the exact parental lineages, but the hybrid origin of *Salsola divaricata* agg. from C_3 and C_4 parental lineages was highly supported.

The network that was recovered most often showed an ancient hybridisation event involving an ancestor of C_4 *Salsola soda* and the C_3 proto-kranz *Salsola montana* giving rise to the *Salsola divaricata* agg. clade. This is the first phylogenomic evidence that a C_3–C_4 intermediate species was the result of (ancient) hybridisation between a C_4 and a C_3 lineage. The second reticulation event later indicated a hybridisation event involving the ancestor of *Salsola divaricata* agg. and the C_4 *Salsola soda* giving rise to the C_4 clade formed by *Salsola oppositifolia*, *Halogeton*, *Hammada* and *Haloxylon*. This might indicate that this relative old C_4 lineage (Salsoleae ~21–22 million years ago (Ma); Morales-Briones *et al.*, 2021), could have inherited C_4 or antecedent traits from a parental lineage, giving them an adaptive advantage that might have influenced their photosynthesis pathway, for example adaptations in photosynthesis or photorespiration-associated enzymes or Kranz cells. This is in line with recent findings in the genus *Flaveria* (Morales-Briones & Kadereit, 2022), in which ancient hybridisation was found to be a step towards C_4 evolution and probably promoted the rapid acquisition of C_4 traits. We also found hybridisation events in the two clades in the other networks with similar parental lineages, supporting the evidence that these lineages were involved in hybridisation events. This could indicate either an incomplete reproductive barrier in the early lineages and recurrent backcrossing or incidences of horizontal gene transfer. We must keep in mind, however, that our sampling is limited and that other lineages not sampled in the C_4 clade might also play a role (please refer to Schüssler *et al.*, 2017 for a plastid tree of the Salsoloideae).

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

A closer look of 50 gene trees from 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 grouped with the C_3 *S. montana* (also the case in the plastid dataset) or with the C_4 species. This pattern could be explained by hybridisation of parental C_3 and C_4 lineages giving rise to an allopolyploid *S. divaricata* agg. with an intermediate expression of

photosynthetic traits. The Kranz-like salsoloid leaf type of the *S. divaricata* agg. (Schüssler *et al.*, 2017: Fig. 7), for example, might occur as for the gene SHORTROOT, a C_4-associated transcription factor that influences the development of Kranz cells (Slewinski *et al.*, 2014; Kelly *et al.*, 2017), with expression of the parental C_4 copy. After 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 C_2 species. For PEPC, two copies were recovered from our transcriptomes and for both the *S. divaricata* agg. group with the C_3 species supporting the C_3-like PEPC activity data found in this study.

Conclusions

The phylogenetic signal in our trees, gene trees and networks indicated that hybridisation events gave rise to the *Salsola divaricata* agg. Furthermore, the distinct variation of three photosynthetic traits within a C_3–C_4 intermediate range without indication of selection towards a more C_4-like expression in this relatively old lineage supported the interpretation of an evolutionarily stable C_2 lineage of hybrid origin. Overall, the photosynthetic traits seemed plastic and genetically fixed stress reactions were not prominent among the populations studied. *Salsola divaricata* agg. is well adapted to a broad range of climatic conditions including mesic to arid coastal habitats and to salinity. Part of the success of the species aggregate might be the photorespiratory pump acquired through hybridisation with a C_4 lineage in the past. However, a broader sampled phylogenomic study is needed to further narrow down the parental lineages. We propose that reticulation events might have played an important role in the multiple convergent acquisition of complex traits such as C_2 or C_4 photosynthesis as a possible fast track route.

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Author contributions

GK designed the study. DTT and GE (carbon isotope values) measured the trait data and DTT analysed them. DTT, DFM-B and YY conducted the phylogenomic analyses. DFM-B prepared the figures and DTT the map. DTT, GK and DFM-B 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

Transcriptome data generated for this study can be found in the NCBI SRA (please refer to Table 1 for SRA accession numbers). Phylogenetic analyses files are available from the Dryad repository 10.5061/dryad.xsj3tx9hh.

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

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

Fig. S2 CO_2 compensation points (Γ in μmol CO_2 mol^{-1}) of populations (P) of the *Salsola divaricata* agg.

Fig. S3 Cladogram of Salsoloideae inferred from the maximum likelihood analyses of the concatenated 991-nuclear gene supermatrix; cladogram of Salsoloideae inferred from the ASTRAL analyses of 991 nuclear gene trees.

Fig. S4 RAxML cladogram of Salsoloideae inferred from the concatenated 991-nuclear gene supermatrix; ASTRAL cladogram of Salsoloideae inferred from 991 nuclear gene 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 hybridisation 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.

Methods S1 More detailed description of methods of CO_2 compensation point measurement, PEPC activity measurement, transcriptome processing and nuclear phylogenetic analyses, assessment of hybridisation, plastome assembly and phylogenetic analysis, and analysis of photosynthetic gene trees.

Table S1 Populations (named by living collection number in the Botanical Garden Mainz; please refer to Fig. 1) included in the carbon isotope measurements, CO_2 compensation point measurements and PEPC activity measurements, including sampling location and collector.

Table S2 Gene and characteristic occupancy of the concatenated matrix.

Table S3 HYDE results from the 298 significant tests.

Table S4 Fifty trees of photosynthesis pathway-associated genes.

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