This is a repository copy of *Photosynthetic diversity in the genus Blepharis (Acanthaceae) and the evolutionary origins of C4 photosynthesis*.

White Rose Research Online URL for this paper:
https://eprints.whiterose.ac.uk/id/eprint/231602/

Version: Accepted Version

Article:

Bianconi, M.E. orcid.org/0000-0002-1585-5947, Osborne, C.P. orcid.org/0000-0002-7423-3718 and Dunning, L.T. orcid.org/0000-0002-4776-9568 (2025) Photosynthetic diversity in the genus Blepharis (Acanthaceae) and the evolutionary origins of C4 photosynthesis. New Phytologist. ISSN: 0028-646X

https://doi.org/10.1111/nph.70541

© 2025 The Author(s). Except as otherwise noted, this author-accepted version of a journal article published in New Phytologist is made available via the University of Sheffield Research Publications and Copyright Policy under the terms of the Creative Commons Attribution 4.0 International License (CC-BY 4.0), which permits unrestricted use, distribution and reproduction in any medium, provided the original work is properly cited. To view a copy of this licence, visit http://creativecommons.org/licenses/by/4.0/

Reuse

This article is distributed under the terms of the Creative Commons Attribution (CC BY) licence. This licence allows you to distribute, remix, tweak, and build upon the work, even commercially, as long as you credit the authors for the original work. More information and the full terms of the licence here: https://creativecommons.org/licenses/

Takedown

If you consider content in White Rose Research Online to be in breach of UK law, please notify us by emailing eprints@whiterose.ac.uk including the URL of the record and the reason for the withdrawal request.

eprints@whiterose.ac.uk
https://eprints.whiterose.ac.uk/

Photosynthetic diversity in the genus *Blepharis* (Acanthaceae) and the evolutionary origins of C_4 photosynthesis

Matheus E. Bianconi[1], Colin P. Osborne[2], Luke T. Dunning[3]

[1] Laboratoire de Recherche en Sciences Végétales (LRSV), Université de Toulouse, CNRS, UPS, Toulouse INP, Castanet-Tolosan, France.
[2] Plants, Photosynthesis and Soil, School of Biosciences, University of Sheffield, Western Bank, Sheffield, S10 2TN, UK.
[3] Ecology and Evolutionary Biology, School of Biosciences, University of Sheffield, Western Bank, Sheffield, S10 2TN, UK.

Word count: 1,322
Number of figures: 1
Number of tables: 0
Number of references: 14

Quote

‘Such a comprehensive phenotyping effort revealed an unexpectedly high diversity in various key C_4 components.’

Main text

Retracing the origins of novel adaptations is a major challenge in evolutionary biology. This problem is best addressed by detailed comparative studies using lineages bearing the ancestral and derived phenotypes. In plants, one of the most well-studied examples is C_4 photosynthesis. The trait has repeatedly emerged in several families of eudicots and monocots through anatomical, ultrastructural and biochemical modifications to the ancestral C_3 physiology that concentrate CO_2 around Rubisco. This mechanism largely suppresses Rubisco’s oxygenase activity, therefore reducing carbon and energy waste from photorespiration. Identifying the key components of the C_4 trait, including their genetic basis and adaptive significance, can advance our understanding of how complex traits emerge during evolution, and is critical for efforts to engineer C_4 photosynthesis into C_3 crops. These goals have been greatly facilitated by genetic and phenotypic studies conducted within an evolutionary framework.

C_4 species are often nested within clades that also contain non-C_4 plants that have adapted to efficiently refix the CO_2 derived from photorespiration. These so-called C_2 plants have C_4-like modifications to leaf structure and gene expression, which together with their close relatedness with

C_4 lineages suggest they represent evolutionary intermediates in the transition to a C_4 physiology (Sage *et al.*, 2012) (although it is important to note the evolutionary stability of 'C_3-C_4 intermediates' in several clades; see Lundgren & Christin, 2017). For this reason, such clades containing C_4 and C_2 species have been chosen as the main models for comparative studies on C_4 evolution. These include the well-studied genera *Flaveria* (Asteraceae) (e.g. Adachi *et al.*, 2023), and the grasses *Alloteropsis* (e.g. Pereira *et al.*, 2023) and *Neurachne* (e.g. Khoshravesh *et al.*, 2020), among others. Together, these studies have revealed a large diversity of C_4-related traits, which have been fundamental to disentangling adaptive changes associated with the C_4 mechanism from other lineage-specific traits. In this issue, Stata et al. contribute to this effort by revealing the vast diversity of photosynthetic traits in the eudicot genus *Blepharis* (Acanthaceae), establishing it as a new model system to understand the evolution of C_4 photosynthesis.

The genus *Blepharis* includes 128 species that occur in seasonally dry and arid areas across Africa, the Middle East and southeastern Asia. The existence of different photosynthetic types in *Blepharis* had been previously reported (Fisher *et al.*, 2015), but the diversity of intermediate phenotypes and their evolutionary significance remained unexplored. To address these gaps, Stata and colleagues conducted an extensive sampling of 29 *Blepharis* species, including several independent populations for some of them, and quantified biochemical, anatomical and leaf-level photosynthesis parameters.

Such a comprehensive phenotyping effort revealed an unexpectedly high diversity in various key C_4 components. To shed light on general patterns across species, the authors used a multivariate statistical approach to discriminate the different samples across physiological space. This suggested that *Blepharis* species can be grouped into five main clusters, which the authors interpreted as different stages along the evolutionary transition from C_3 to C_4 physiology. The first cluster corresponded to typical C_3 species with low activity of C_4-related enzymes, such as phosphoenolpyruvate carboxylase (PEPC) and pyruvate phosphate dikinase (PPDK), and CO_2 compensation point (CCP) above 50 μmol mol^{-1}. This group is sister to the remaining C_4 and intermediate lineages, and likely retains the photosynthetic characteristics of the common ancestor of all Acanthaceae. A second well-defined group corresponded to the C_4 species, which exhibited the highest activity of C_4-related enzymes, the lowest CCP values, and carbon isotope signatures ($\delta^{13}C$) that are diagnostic of C_4 plants. Surprisingly, the phylogenetic distribution of these species suggested five independent C_4 clades in *Blepharis*, which is the highest number of transitions to the C_4 state so far reported for a single genus. The remaining species were assigned to three additional categories. The first was to a large extent similar to the C_4 type, although with significantly lower PEPC activity and lower leaf-level carboxylation efficiency, which were associated with $\delta^{13}C$ values at the upper limit of usual C_4 values. The three species displaying this phenotype were classified as C_4-, what the authors described as species with an incomplete expression of C_4 characters, such as absence of a full compartmentalization of Rubisco to the bundle sheath tissue. The two other categories included all species with key diagnostic features of C_2 plants, including the restriction of GDC to the bundle sheath tissue, and intermediate CCP values between C_3 and C_4 plants. One of the C_2 groups, named as C_2+, was composed by a single species, *B. mitrata*, which displayed higher activity of some C_4-related enzymes and low CCP, although with C_3-like $\delta^{13}C$ values.

One particular aspect of this study is the large intraspecific variation in $\delta^{13}C$ observed in herbarium specimens of three species - *B. furcata*, *B. macra* and *B. mitrata* -, with values spanning typical C_3 and C_4 values. This suggests that the three species contain recently diverged C_4 and non-C_4 populations, a finding that has been so far limited to the grass *Alloteropsis semialata*. Although a phylogenetic assessment of the specimens analysed is necessary to rule out taxonomic issues, this would not be surprising given the high diversity in C_4-related traits in *Blepharis*. This highlights the importance of sampling multiple populations for trait evolution studies, particularly in cases of complex traits such as C_4 photosynthesis.

C_4 research has diversified over the past twenty years from physiological and anatomical descriptions into phylogenetically controlled investigations, often accompanied by genetic insights from comparative genomic and transcriptomic analyses. This has created a broad understanding of the circumstances of C_4 repeated evolution, and was fundamental for developing the initial strategies to engineer the C_4 trait in C_3 crops. However, two major gaps in our knowledge still remain. First, although some regulatory mutations linked to C_4 anatomy and gene expression have been identified (e.g. Swift *et al.*, 2024; Vlad *et al.*, 2025), our knowledge of the genetic control of the C_4 trait is largely limited to a few models, particularly maize. The second major gap is the lack of a consensus on the minimum set of components necessary for a full C_4 physiology. The latter in particular is a difficult task because the phenotype we observe in living species is a result of lineage-specific constraints and increments, that might be adaptive or not, and might have evolved after the trait was first put in place. The genus *Blepharis* provides a new opportunity to reassess these problems under a new perspective.

Another important shift in C_4 research regards the adoption of experimental strategies using controlled crosses and genetic transformation. These are powerful tools not only to dissect the genetic basis of C_4 components (Simpson *et al.*, 2022), but also to experimentally investigate longstanding hypotheses on C_4 evolution from modelling studies (e.g. Mallmann *et al.*, 2014), an effort that has been long missing. Nonetheless, progress has been slow due to the current lack of an ideal experimental model. In the grass *A. semialata*, for example, which includes closely related C_3, C_4 and intermediates, although F1 photosynthetic hybrids were obtained (Bianconi *et al.*, 2022), the generation of segregating F2 hybrids has been proven challenging. Likewise, tissue culture protocols for the species have not advanced, which limits the implementation of genetic transformation approaches. Nonetheless, population-level genome sequencing across the species range allowed a genome-wide association study (GWAS) that identified several genomic regions underlying C_4 features (Alenazi *et al.*, 2024). The genus *Blepharis*, particularly if C_2 and C_4 phenotypes are confirmed within *B. furcata*, *B. macra* and *B. mitrata*, provides a new opportunity for GWAS and genetic approaches to identify loci controlling C_4 traits. Similarly, crossing between photosynthetic types could also be explored, although different chromosome number among species has been reported to the genus (Ranganath & Krishnappa, 1982), which might complicate such efforts. Finally, the fast germination and growth rates as Stata et al. reported for some *Blepharis* species are desirable characteristics for a genetic model, and these might provide a promising alternative for establishing such a long-awaited model for experimental investigations.

References

Adachi S, Stata M, Martin DG, Cheng S, Liu H, Zhu XG, Sage RF. 2023. The evolution of C4 photosynthesis in Flaveria (Asteraceae): Insights from the Flaveria linearis complex. Plant Physiology, 191: 233–251. https://doi.org/10.1093/plphys/kiac467

Alenazi AS, Pereira L, Christin P-A, Osborne CP, Dunning LT. 2024. Identifying genomic regions associated with C4 photosynthetic activity and leaf anatomy in Alloteropsis semialata. New Phytologist 243: 1698-1710. https://doi.org/10.1111/nph.19933

Bianconi ME, Sotelo G, Curran EV, Milenkovic V, Samaritani E, Dunning LT, Bertolino LT, Osborne CP, Christin P-A. 2022. Upregulation of C4 characteristics does not consistently improve photosynthetic performance in intraspecific hybrids of a grass. Plant, Cell & Environment 45: 1398-1411. https://doi.org/10.1111/pce.14301

Fisher AE, McDade LA, Kiel CA, Khoshravesh R, Johnson MA, Stata M, Sage TL, Sage RF. 2015. Evolutionary history of Blepharis (Acanthaceae) and the origin of C4 photosynthesis in section Acanthodium. International Journal of Plant Sciences, 176: 770–790. https://doi.org/10.1086/683011

Khoshravesh R, Stata M, Busch FA, Saladié M, Castelli JM, Dakin N, Hattersley PW, MacFarlane TD, Sage RF, Ludwig M, Sage TL. 2020. The evolutionary origin of C4 photosynthesis in the grass subtribe neurachninae. Plant Physiology, 182: 566–583. https://doi.org/10.1104/pp.19.00925

Lundgren MR, Christin P-A. 2017. Despite phylogenetic effects, C3–C4 lineages bridge the ecological gap to C4 photosynthesis. Journal of Experimental Botany 68: 241–254. https://doi.org/10.1093/jxb/erw451

Mallmann J, Heckmann D, Bräutigam A, Lercher MJ, Weber APM, Westhoff P, Gowik U. 2014. The role of photorespiration during the evolution of C4 photosynthesis in the genus Flaveria. eLife 3:e02478. https://doi.org/10.7554/eLife.02478

Pereira L, Bianconi ME, Osborne CP, Christin P-A, Dunning LT. 2023. Alloteropsis semialata as a study system for C4 evolution in grasses. Annals of Botany 132: 365–382. https://doi.org/10.1093/aob/mcad078

Ranganath RM, Krishnappa DG. 1982. Telocentric chromosomes and karyotypes in the genus Blepharis. Caryologia 35: 237-246. https://doi.org/10.1080/00087114.1982.10796928

Sage RF, Sage TL, Kocacinar F. 2012. Photorespiration and the evolution of C4 photosynthesis. Annual Review of Plant Biology 63: 19–47. https://doi.org/doi:10.1146/annurev-arplant-042811-105511

Simpson CJC, Reeves G, Tripathi A, Singh P, Hibberd JM. 2022. Using breeding and quantitative genetics to understand the C4 pathway. Journal of Experimental Botany 73: 3072–3084. https://doi.org/10.1093/jxb/erab486

Stata M, Lyu M-JA, Liu H, Cheng S, Zhu X-G, Sage TL, Sage RF. 2025. How evolution repeatedly builds complexity: A case study with C4 photosynthesis in Blepharis (Acanthaceae). New Phytologist 00-00-00

Swift J, Luginbuehl LH, Hua L, Schreier TB, Donald RM, Stanley S, Wang N, Lee TA, Nery JR, Ecker JR, Hibberd JM. 2024. Exaptation of ancestral cell-identity networks enables C4 photosynthesis. Nature 636. https://doi.org/10.1038/s41586-024-08204-3

Vlad D, Zaidem M, Perico C, Sedelnikova O, Bhattacharya S, Langdale JA. 2024. The WIP6 transcription factor TOO MANY LATERALS specifies vein type in C4 and C3 grass leaves. Current Biology 34: 1670-1686.e10. https://doi.org/10.1016/j.cub.2024.03.007

Keywords (5-8): photorespiration, C_3-C_4 intermediates, C_2 photosynthesis, complex traits, phylogenetics, leaf anatomy, Rubisco, glycine decarboxylase.

Figures, tables and boxes

Figure 1. Diversity of photosynthetic phenotypes in the genus *Blepharis* (Acanthaceae). Phylogeny redrawn from Stata et al. (2025), with representative species shown for each clade. Photographs of *Blepharis* species reproduced with permission from Stata et al. (2025).