

SHORT COMMUNICATION

## Molecular phylogenies disprove a hypothesized C<sub>4</sub> reversion in *Eragrostis walteri* (Poaceae)

Amanda L. Ingram<sup>1,\*</sup>, Pascal-Antoine Christin<sup>2,3</sup> and Colin P. Osborne<sup>4</sup>

<sup>1</sup>Department of Biology, Wabash College, Crawfordsville, IN 47933, USA, <sup>2</sup>Department of Ecology and Evolution, University of Lausanne, 1015 Lausanne, Switzerland, <sup>3</sup>Department of Ecology and Evolutionary Biology, Brown University, Providence, RI 02912, USA and <sup>4</sup>Department of Animal and Plant Sciences, University of Sheffield, Sheffield S10 2TN, UK

\*For correspondence. E-mail [ingrama@wabash.edu](mailto:ingrama@wabash.edu)

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- **Background and Aims** The main assemblage of the grass subfamily Chloridoideae is the largest known clade of C<sub>4</sub> plant species, with the notable exception of *Eragrostis walteri* Pilg., whose leaf anatomy has been described as typical of C<sub>3</sub> plants. *Eragrostis walteri* is therefore classically hypothesized to represent an exceptional example of evolutionary reversion from C<sub>4</sub> to C<sub>3</sub> photosynthesis. Here this hypothesis is tested by verifying the photosynthetic type of *E. walteri* and its classification.
- **Methods** Carbon isotope analyses were used to determine the photosynthetic pathway of several *E. walteri* accessions, and phylogenetic analyses of plastid *rbcl* and *ndhF* and nuclear internal transcribed spacer DNA sequences were used to establish the phylogenetic position of the species.
- **Results** Carbon isotope analyses confirmed that *E. walteri* is a C<sub>3</sub> plant. However, phylogenetic analyses demonstrate that this species has been misclassified, showing that *E. walteri* is positioned outside Chloridoideae in Arundinoideae, a subfamily comprised entirely of C<sub>3</sub> species.
- **Conclusions** The long-standing hypothesis of C<sub>4</sub> to C<sub>3</sub> reversion in *E. walteri* is rejected, and the classification of this species needs to be re-evaluated.

**Key words:** C<sub>4</sub> photosynthesis, evolution, reversion, *Eragrostis*, Chloridoideae, Arundinoideae, Poaceae, Africa, Namibia.

### INTRODUCTION

Complex traits have received a great deal of attention by evolutionary biologists, but key questions remain regarding the directionality of transitions between their states and, in particular, about their reversibility (e.g. Collin and Miglietta, 2008; Tripp and Manos, 2008; Lynch and Wagner, 2009). C<sub>4</sub> photosynthesis is a prime example of a complex trait due to the numerous morphological, anatomical and biochemical adaptations relative to ancestral C<sub>3</sub> photosynthesis that are required for proper function. These adaptations are thought to involve hundreds of genetic changes (Bräutigam *et al.*, 2010), but nonetheless have been demonstrated to be evolutionarily labile. C<sub>4</sub> photosynthesis has evolved numerous times independently in distantly related plant families during the past 30 million years, with >50 independent origins inferred in the angiosperms (Giussani *et al.*, 2001; Kadereit *et al.*, 2003; Sage, 2004; Muhaidat *et al.*, 2007; Christin *et al.*, 2008; Besnard *et al.*, 2009). The majority of C<sub>4</sub> plant species belong to the grass family, in which C<sub>4</sub> taxa form a minimum of 17 different lineages separated in the phylogeny by C<sub>3</sub> taxa within the PACMAD clade (Panicoideae, Arundinoideae, Chloridoideae, Micrairoideae, Aristidoideae and Danthonioideae; Sage, 2004; Christin *et al.*, 2008). Comparisons of the phenotype and genotype of the C<sub>4</sub> traits used by these different C<sub>4</sub> phylogenetic groups in grasses suggest that most derive from independent C<sub>4</sub> origins (Christin *et al.*, 2010), ranking C<sub>4</sub> photosynthesis amongst the most convergent of complex traits (Conway-Morris, 2006). Surprisingly,

very few putative losses of the C<sub>4</sub> pathway and recovery of the ancestral C<sub>3</sub> trait have been identified. Two exceptions are *Alloteropsis semialata* (R.Br.) Hitchc. subsp. *eckloniana* (Nees) Gibbs Russ., a C<sub>3</sub> subspecies nested in a C<sub>4</sub> clade of Panicoideae (Ibrahim *et al.*, 2009), and *Eragrostis walteri* Pilg.

*Eragrostis walteri* is a grass endemic to Namibia that was described in 1941 (Pilger, 1941) and that possesses many morphological features typical of *Eragrostis* species, including multifloreted spikelets, paniculate inflorescences and ciliate ligules. This placement within *Eragrostis* was confirmed by phylogenetic analyses of morphological data (van den Borre and Watson, 1994). *Eragrostis* contains about 400 species distributed worldwide and is placed in the main assemblage of Chloridoideae, which contains >1400 species. Numerous Chloridoideae species have been studied for leaf anatomy and/or carbon isotope composition, and all have been found to be C<sub>4</sub>, making this group the largest wholly C<sub>4</sub> clade in plants. This fact made the report of non-C<sub>4</sub> leaf anatomy in *E. walteri* by Ellis (1984) particularly striking. The photosynthetic pathway employed by Ellis' *E. walteri* specimens was subsequently confirmed by  $\delta^{13}\text{C}$  analysis (Schulze *et al.*, 1996). The presence of a C<sub>3</sub> plant in an otherwise C<sub>4</sub> clade was strongly suggestive of an evolutionary loss of C<sub>4</sub> photosynthesis. Consequently, *E. walteri* has been repeatedly cited as the best candidate for C<sub>4</sub> to C<sub>3</sub> reversion for the last 25 years (e.g. Renvoize, 1987; Morrone and Zuloaga, 1991; Kellogg, 1999; Kubien *et al.*, 2008; Ibrahim *et al.*, 2009; Edwards and Smith, 2010; Roalson, 2011).

Given that the leaf anatomy of *E. walteri* shows no evidence of partially  $C_4$  characters, confirmation of this putative  $C_4$  to  $C_3$  reversion in *Eragrostis* would demonstrate that  $C_4$  evolution is reversible. This case is particularly remarkable because the reversion would probably have occurred tens of millions of years after the initial  $C_4$  origin in Chloridoideae, which is estimated to have occurred between 25 and 32 million years ago (Christin *et al.*, 2008). Furthermore, the Chloridoideae encompasses numerous species with well-optimized  $C_4$  characters that confer ecological success in many of the world's biomes. If the hypothesis of reversion from  $C_4$  to  $C_3$  photosynthesis was proven, *E. walteri* would represent an outstanding system in which to investigate the genetic mechanisms and ecological pressures involved (Christin *et al.*, 2010). However, it is necessary first to confirm that *E. walteri* does in fact use  $C_3$  photosynthesis with multiple independent collections of the species. Additionally, the assumption that *E. walteri* is nested in a  $C_4$  clade relies solely on morphological evidence that has not been confirmed with genetic markers. This is crucial, since polyphyly has been demonstrated for several grass genera upon phylogenetic analysis of genetic data (e.g. Aliscioni *et al.*, 2003; Peterson *et al.*, 2010).

In this study, we investigated the likelihood of an evolutionary reversion from  $C_4$  to  $C_3$  photosynthesis in *E. walteri* to gain insights into the reversibility of  $C_4$  evolution. To test the hypothesis of reversion, our work aimed to: (a) verify the photosynthetic type of several *E. walteri* specimens using unambiguous methods; and (b) determine the phylogenetic position of *E. walteri* using genetic markers from the plastid and nuclear genomes.

## MATERIALS AND METHODS

### Carbon isotope ratio

The  $C_4$  pathway is defined by the fixation of atmospheric  $CO_2$  through a coupling of carbonic anhydrase and phosphoenolpyruvate carboxylase, whereas in  $C_3$  plants this fixation is performed by ribulose-1,5-bisphosphate carboxylase. These enzymes differentially discriminate between the carbon isotopes naturally present in the atmosphere, resulting in different ratios of carbon isotopes in the plants that can be determined by mass spectrometry. Values of  $\delta^{13}C$  between  $-21\text{‰}$  and  $-32\text{‰}$  are indicative of  $C_3$  photosynthesis, while  $C_4$  plants have  $\delta^{13}C$  between  $-9\text{‰}$  and  $-16\text{‰}$ . Some well-developed  $C_3$ – $C_4$  intermediates can have  $\delta^{13}C$  values between  $-16\text{‰}$  and  $-19\text{‰}$ , but there is no overlap between the  $\delta^{13}C$  values of wholly  $C_3$  and  $C_4$  species (von Caemmerer, 1992).

Foliar  $\delta^{13}C$  values were determined on six herbarium samples whose identification as *Eragrostis walteri* had been verified by T. A. Cope and M. Vorontsova by reference to the specimen collection held at RBG Kew. A 20 mg subsample from each was analysed using an ANCA GSL preparation module coupled to a 20–20 stable isotope analyser (PDZ Europa, Cheshire, UK). Measurements on the same sample had a reproducibility of 0.5‰, and the isotopic composition of each ( $\delta^{13}C$ ) was calculated as the sample  $^{13}C/^{12}C$  ratio relative to the PDB standard (‰).

### Molecular phylogenies

Two herbarium specimens of *E. walteri* (K:Kolberg & Tholkes 695 and PRE:Hines 262) were selected for genetic analyses. The Kolberg & Tholkes 695 sample was analysed in Lausanne, while the Hines 262 sample was analysed at Wabash College. No laboratory products or PCR primers were shared between these laboratories, excluding the possibility of contamination. At Lausanne, two plastid markers (*rbcL* and *ndhF*) were amplified and sequenced using a previously published methodology (Christin *et al.*, 2008). At Wabash College, *rbcL* was amplified and sequenced with new primers designed specifically for grasses: 7F (5'-GGGACTTATGTCACCACAAAC-3') and 1433R (5'-AC TTAATCGATGGTATCTACCG-3'). Amplifications were carried out as described in Ingram and Doyle (2003) with an annealing temperature of 55 °C. DNA sequencing was completed by the Cornell BioResource Center. All plastid sequences were added to a previously published data set with representatives of all Poaceae subfamilies (Christin *et al.*, 2008). Phylogenetic methods are as previously described (Christin *et al.*, 2008). An independent estimate of phylogeny was obtained with sequences from the nuclear ribosomal DNA internal transcribed spacer (ITS). New grass-specific ITS primers were used for amplification and sequencing [ITS 18S-F-grass (5'-ATTGAATGGTCCGGTGAA G-3') and ITS 26S-R-grass (5'-GACGCCTCTCCAGACTAC AA-3')]. PCR was as described in Ingram and Doyle (2003) with annealing temperatures of 56 °C. ITS PCR products were sequenced directly. An ITS data set was assembled from sequences deposited in GenBank, which contains several thousand ITS sequences for grasses. The selected sequences included numerous representatives of all PACMAD families, with Pooideae outgroups. Details on the species and GenBank accession numbers can be found in the Supplementary Data (available online). The sequences were aligned with ClustalX (Larkin *et al.*, 2007), and a phylogenetic tree was inferred as described for the plastid markers (Christin *et al.*, 2008). All *E. walteri* sequences were deposited in GenBank (accession numbers HQ329788–HQ329791).

## RESULTS AND DISCUSSION

The  $\delta^{13}C$  values (Table 1) ranged between  $-24.3\text{‰}$  and  $-29.1\text{‰}$  for the six *E. walteri* accessions, which unambiguously indicates that these plants assimilated carbon via  $C_3$  photosynthesis. This confirms previous conclusions (Ellis, 1984; Schulze *et al.*, 1996) with independent samples of *E. walteri*.

The *rbcL* sequences for *E. walteri* obtained independently in the two different laboratories were identical. In the phylogeny, however, they did not group with Chloridoideae as expected from morphology (van den Borre and Watson, 1994). Instead, *E. walteri* was placed within the Arundinoideae, sister to a clade composed of *Molinia* and *Phragmites* (Fig. 1). This position within Arundinoideae was strongly supported (Bayesian support values  $>0.95$ ). Blasting the *E. walteri* ITS sequence showed the highest similarity with *Molinia caerulea* (94%) followed by *Phragmites* spp. (90–91%). The Bayesian inference confirmed this close

relationship between *E. walteri* ITS and those of *Molinia* and *Phragmites* (Fig. 2), congruent with the results from the plastid markers. This relationship was also highly supported (Bayesian support value of 1.0). Therefore, both nuclear and plastid markers show that *E. walteri* does not belong to *Eragrostis*, nor to Chloridoideae, but is unambiguously a member of Arundinoideae. The positioning of *E. walteri* outside *Eragrostis* is not entirely surprising when morphological features are more carefully examined. *Eragrostis* is a highly heterogeneous group, but *E. walteri* is an outlier in some otherwise invariable traits. For example, *Eragrostis* lemmas are consistently three-nerved, but Pilger (1941) noted in his original description of *E. walteri* that this species has three prominent and two inconspicuous nerves on the lemmas. In addition, *E. walteri* lemma apices have been described as

‘nearly awned’ (Watson and Dallwitz, 1992 onwards), in contrast to the acute lemma apices found in most other species in the genus. The discrepancy between morphological classification and molecular phylogenies mirrors the numerous cases of polyphyletic genera in grasses (e.g. Giussani *et al.*, 2001; Aliscioni *et al.*, 2003; Peterson *et al.*, 2010).

Because molecular data indicate that *E. walteri* belongs to Arundinoideae, its C<sub>3</sub> type is no longer surprising, as all other species of Arundinoideae are also C<sub>3</sub>. Therefore, the hypothesis that this species is a C<sub>4</sub> to C<sub>3</sub> revertant should be abandoned. With our current understanding of grass phylogenetics and photosynthetic pathways, *Alloteropsis semialata* subsp. *eckloniana* is the only plausible C<sub>4</sub> revertant in grasses and should now be more closely investigated to detect traces of C<sub>4</sub> loss (Christin *et al.*, 2010). However, this new discovery regarding the misclassification of *E. walteri* clearly demonstrates the dominance of C<sub>3</sub> to C<sub>4</sub> transitions over reversions, suggesting that C<sub>4</sub> evolution is almost always a one-way event. The resolution of the enigma created by the peculiar foliar anatomy of *E. walteri* also highlights the importance of working with species or even accessions as evolutionary units, and the risks of extrapolating phylogenetic positions from congeners. While grass phylogeny is far from being resolved at the species level, efforts should be put into incorporating as many of the evolutionarily interesting taxa as possible, until an exhaustive phylogeny is obtained. This could reveal other surprises, including the non-monophyly of numerous morphological taxonomic units, even at the subfamily level, as for *E. walteri*.

TABLE 1. Stable carbon isotope ratio ( $\delta^{13}C$ ) for leaf material of *Eragrostis walteri* (Pilg.)

Collector and collection number	$\delta^{13}C$
Giess, W. 8977	-27.3
Giess, W. 8104A	-25.8
Kolberg, H. and Tholkes, T. 695	-28.2
Giess, W. 10413	-28.1
Giess, W. and Müller, M. 14316	-29.1
Range, P. 14831	-24.3

All specimens were collected in southern Africa and were identified and archived in the herbarium of the Royal Botanic Gardens, Kew.

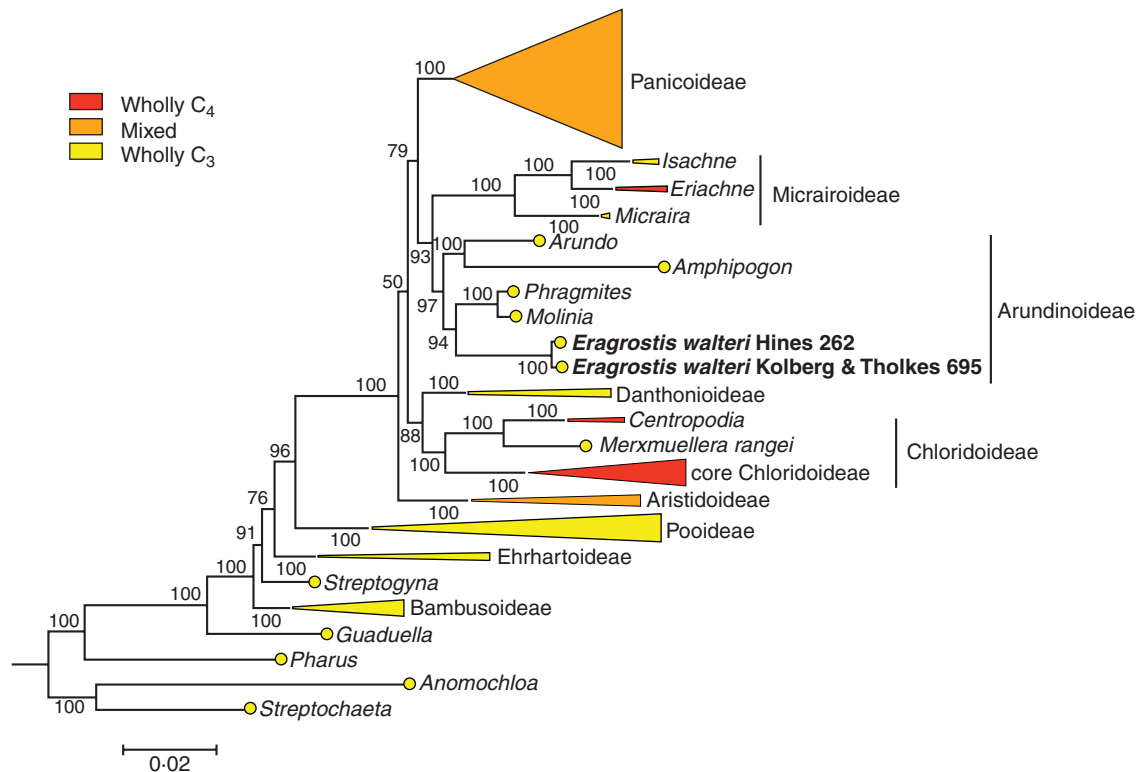


FIG. 1. Phylogenetic position of *E. walteri* inferred from plastid markers. This tree was obtained through Bayesian inference based on *ndhF* and *rbcl* sequences. Bayesian support values are indicated near nodes. The main groups are compressed. Clades containing only C<sub>3</sub> taxa are in yellow, those containing only C<sub>4</sub> taxa are in red, and those containing both C<sub>3</sub> and C<sub>4</sub> taxa are in orange. For further details on the data set see Christin *et al.* (2008).

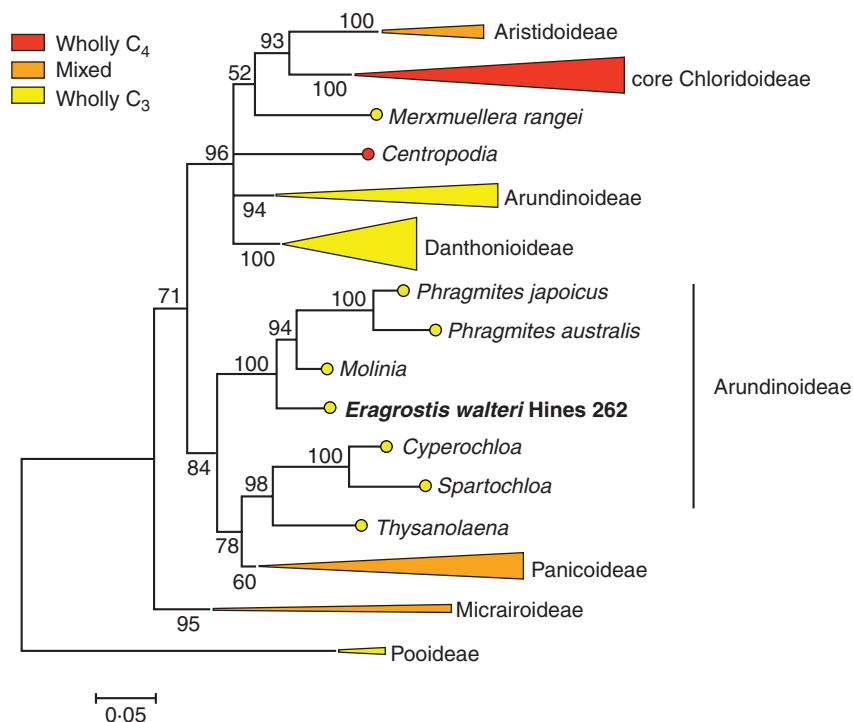


FIG. 2. Phylogenetic position of *E. walteri* inferred from nuclear markers. This tree was obtained through Bayesian inference based on nuclear ribosomal DNA internal transcribed spacer sequence. Bayesian support values are indicated near nodes. The main groups are compressed, and their delimitation follows Christin *et al.* (2008) and Fig. 1. Clades are coloured as in Fig. 1.

#### SUPPLEMENTARY DATA

Supplementary data are available online at [www.aob.oxfordjournals.org](http://www.aob.oxfordjournals.org) and give the GenBank accession numbers of the species used in the phylogenetic analysis of ITS data.

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