Multiple photosynthetic transitions, polyploidy, and lateral gene transfer in the grass subtribe Neurachninae

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Abstract

The Neurachninae is the only grass lineage known to contain C3, C4, and C3–C4 intermediate species, and as such has been suggested as a model system for studies of photosynthetic pathway evolution in the Poaceae; however, a lack of a robust phylogenetic framework has hindered this possibility. In this study, plastid and nuclear markers were used to reconstruct evolutionary relationships among Neurachninae species. In addition, photosynthetic types were determined with carbon isotope ratios, and genome sizes with flow cytometry. A high frequency of autopolyploidy was found in the Neurachninae, including in Neurachne munroi F.Muell. and Paraneurachne muelleri S.T.Blake, which independently evolved C4 photosynthesis. Phylogenetic analyses also showed that following their separate C4 origins, these two taxa exchanged a gene encoding the C4 form of phosphoenolpyruvate carboxylase. The C3–C4 intermediate Neurachne minor S.T.Blake is phylogenetically distinct from the two C4 lineages, indicating that intermediacy in this species evolved separately from transitional stages preceding C4 origins. The Neurachninae shows a substantial capacity to evolve new photosynthetic pathways repeatedly. Enablers of these transitions might include anatomical pre-conditions in the C3 ancestor, and frequent autopolyploidization. Transfer of key C4 genetic elements between independently evolved C4 taxa may have also facilitated a rapid adaptation of photosynthesis in these grasses that had to survive in the harsh climate appearing during the late Pliocene in Australia.

Keywords: C3 grass evolution, C4 photosynthesis, C3–C4 intermediate, grass phylogeny, lateral gene transfer, Neurachne, Paraneurachne, polyploidy

Introduction

Despite its complexity, the C4 photosynthetic pathway has evolved independently >62 times in flowering plants (Sage et al., 2011), thus constituting a striking example of convergent evolution. It is especially prevalent in grasses, where 22–24 distinct C4 lineages have been postulated in the PACMAD clade (Grass Phylogeny Working Group II, 2012). Such a clustering of C4 origins is also observed in other groups, with six independent lineages in the sedges (Cyperaceae) and 23 in the Caryophyllales.
(Sage et al., 2011). These patterns indicate that some plant groups have a higher propensity for C_4 photosynthesis evolution, which may reflect ecological, genomic, and/or anatomical factors that facilitate the acquisition of novel traits (Sage, 2001; Marshall et al., 2007; McKown and Dengler, 2007; Christin et al., 2011; Edwards and Ogburn, 2012). Leading environmental factors promoting C_4 evolution are low atmospheric CO_2, heat, drought and salinity, often in combination (Sage et al., 2012). Anatomical factors include high vein density, which may be common in dry environments and certain taxonomic groups such as the grasses (Ehleringer et al., 1979; Sage et al., 2012).

The evolution of C_4 photosynthesis is best studied using closely related taxa with different photosynthetic types, as in the eudicot groups Flaveria, Cleome, Molluginaceae, and Heliotropium (McKown et al., 2005; Marshall et al., 2007; Feodorova et al., 2010; Christin et al., 2011; Muha et al., 2011). These groups, however, have limited utility for understanding the origins of the pathway in the grasses, where half of all C_4 species occur. C_4 grasses are the most successful group of C_4 plants on Earth, dominating the 23% of global primary productivity attributable to C_4 vegetation, and comprising the vast majority of C_4 plants in agricultural use (Brown, 1999; Still et al., 2003). Efforts to engineer the C_4 pathway into C_3 crops to take advantage of the superior productivity of C_4 photosynthesis have been directed towards the grasses rice and wheat (http://irri.org/c4rice); however, these efforts are hindered by the lack of a model group for studying C_4 evolution in the Poaceae. Such a model group could be exploited to identify the genetic changes that occurred during C_4 evolution, as well as elucidating the order in which the individual traits of the pathway were assembled. The major C_4 lineages of grasses (e.g. Chloridoideae and Andropogoneae) are composed of numerous and ecologically successful C_4 species, but lack C_3–C_4 intermediate species, and are only distantly related to C_3 taxa (Christin et al., 2008; Grass Phylogeny Working Group II, 2012). Photosynthetic variation exists in some small groups of grasses, notably Steinchisma, which contains C_3–C_4 intermediates and C_3 species, and Alloteropsis, which has both C_3 and C_4 taxa (Duvall et al., 2003; Ibrahim et al., 2009; Christin et al., 2010), but only one grass clade has been identified that contains C_3, C_4, and C_3–C_4 intermediate species. This is the Neurachne/Thyridolepis clade (in the subtribe Neurachninæ; Morrone et al., 2012), a group of three genera and 11 species endemic to Australia (Blake, 1972; Macfarlane, 2007).

Within the Neurachninæ, the genus Neurachne includes one C_4 species (N. munroi), five C_3 species, and one C_3–C_4 intermediate species, N. minor (Hattersley et al., 1982, 1986; Hattersley and Roksandic, 1983; Macfarlane, 2007). The monospecific genus Paraneurachne (P. muelleri) is C_4, while the other genera (Thyridolepis, Ancistrachne, Cleistochloa, and Calyptochloa) are C_3. The clade belongs to the Panicoidae subfamily, which encompasses the vast majority of C_4 grass lineages, and thus appears especially prone to transitions from C_3 to C_4 biochemistry (Grass Phylogeny Working Group II, 2012). Extensive work in the 1980s characterized the anatomy, biochemistry, and physiology of Neurachninæ species (Hattersley et al., 1982, 1986; Hattersley and Roksandic, 1983; Hattersley and Stone, 1986; Moore and Edwards, 1989), indicating high potential for this group to serve as a model for C_4 grass evolution. However, to make evolutionary inferences, it is necessary to have a well-resolved, species-level phylogeny. Such a phylogeny was not available for the Neurachninæ, as only some members of the group have been analysed with a small number of molecular markers (Hudson et al., 1990; Christin et al., 2008; Grass Phylogeny Working Group II, 2012).

The objective of the present study was a reconstruction of the evolutionary history of the Neurachninæ, with an emphasis on photosynthetic pathway evolution. Multiple accessions per species were sampled, and phylogenetic analyses of plastid as well as nuclear markers, photosynthetic pathway identification, and measures of genome size were included. This comparative approach established the phylogenetic relationships between Neurachninæ species, and revealed the genome dynamics of the group. The outcomes also highlight the diversity of photosynthetic transitions in the Neurachninæ, and provide a solid foundation for future studies aimed at elucidating the anatomical and molecular mechanisms underlying these transitions.

Materials and methods

Plant material

Live, field-collected individuals of Neurachninæ species were sampled for genome size analyses using flow cytometry (Supplementary Table S1 available at JXB online), while carbon isotope ratios were determined using multiple herbarium samples of each species (Supplementary Appendix S1). Multiple individuals of each Neurachninæ species were also sampled for phylogenetic analyses; these were herbarium specimens or plants collected from the field (Supplementary Tables S1, S2).

Carbon isotope ratios (δ^{13}C)

Carbon isotope values were determined for all described Neurachne, Paraneurachne, and Thyridolepis species (Supplementary Appendix S1 at JXB online) by the University of California, Davis Stable Isotope Facility (http://stableisotop facility.ucdavis.edu). Some of the samples were previously assayed for δ^{13}C (Hattersley and Roksandic, 1983; Hattersley et al., 1986).

Determination of genome size

Nuclei were simultaneously released from fresh leaf material from a Neurachninæ species and a calibration standard [Raphanus sativus cv. Saxa (2C DNA content = 1.11 pg; Doležel et al., 1992) or Lepidoperma gibsonii (2C DNA content = 0.56 pg; M. Wallace, unpublished results)] by chopping with a razor blade in cold buffer (Roberts, 2007). Samples were filtered, and the nuclei were stained with propidium iodide (Roberts, 2007) and analysed using a BD FACSCanto II flow cytomter (Becton Dickinson, Franklin Lakes, NJ, USA) with 488 nm excitation and 585/424 band pass filter detection. Data were collected with BD FACS DIVA software (v. 5.0.2) and analysed using FlowJo v.7.6.3 software (Tree Star Inc., Ashland, OR, USA). At least 5000 nuclei per day were analysed from each individual on three separate days. The genome size of an individual was then calculated as the average of these three estimates. Genome size and standard deviation (SD) of conspecific homopoloids (Supplementary Table S1 at JXB online) were calculated using the average genome sizes of the individual homopoloids. Estimates were only included if the peak heights of the sample and standard were similar, and the coefficient of variation was <5%. Conversion of pg DNA to Mbp DNA followed Doležel et al. (2003).

Amplification of plastid markers and ITS

Five plastid markers (ndhF, trnK/matK, rpoC2, rpl16, and rnlL) that have been used previously to investigate relationships among grasses...
Multiple photosynthetic transitions in the Neurachninae | 6299

(Duvall et al., 2003; Salariento et al., 2010; Grass Phylogeny Working Group II, 2012) were amplified from genomic DNA (gDNA) extracted from herbarium samples (MP FastDNA SPIN Kit and FastPrep Instrument; MP Biomedicals LLC, Solon, OH, USA). For ndhF and trnK/matK, previously published primers were used (Grass Phylogeny Working Group II, 2012), while primers for the three other markers were designed in the conserved regions of Panicoideae sequences downloaded from GenBank (Supplementary Table S3 at JXB online). The nuclear region encompassing the internal transcribed spacer 1, 5.8S rRNA, and internal transcribed spacer 2 (ITS) was amplified with the universal primers ITS4_rev and ITS5_for (Supplementary Table S3; White et al., 1990). However, in half the samples, these primers amplified endophytic fungal genes. A new forward primer, specific to grasses (ITS_grasses_for; Supplementary Table S3), was then used in combination with the ITS4 reverse primer. The plastid and ITS markers were amplified in overlapping fragments of ~300–700 bp, with the protocol described in Grass Phylogeny Working Group II (2012). Single nucleotide polymorphisms were detected in most of the ITS sequences. These were coded as ambiguous characters in the phylogenetic analyses. All sequences have been submitted to GenBank (Supplementary Table S2).

Amplification of low-copy nuclear markers

As the gDNA extracted from herbarium specimens was too degraded to amplify low-copy nuclear markers, gDNA was isolated (DNeasy Plant Mini Kit, QIAGEN, Hilden, Germany) from selected accessions for which fresh material was available (Supplementary Table S4 at JXB online). Fragments of the genes encoding waxy and ardoeh were amplified with previously designed primers (Christin et al., 2012b). The ppc-B2 lineage of phosphoenolpyruvate carboxylase (PEPC), which contains most C$_4$-specific forms in grasses as well as non-C$_4$ orthologues (Christin et al., 2007), was isolated in one fragment or two overlapping segments, using previously published primers (Christin et al., 2007, 2012b). These three markers were isolated as described previously (Christin et al., 2012b), but with an annealing temperature of 51 °C and an extension time of 2 min. All sequences have been submitted to GenBank (Supplementary Table S4).

Phylogenetic analyses

The veracity of the sequence data was monitored throughout the analyses. The congruence between accessions of the same species and the lack of identical sequences for any of the plastid or nuclear markers, including the ppc-B2 genes, from different Neurachninae species indicated no cross-contamination or misidentification of the samples.

Sequences were aligned with MUSCLE (Edgar, 2004), and the alignments were manually refined. Outgroups were included based on previous work (Christin et al., 2012b; Grass Phylogeny Working Group II, 2012). Best substitution models were determined by hierarchical likelihood ratio tests. In all cases, the general time reversible substitution model with a gamma shape parameter (GTR+G) was selected. Phylogenetic analyses were carried out from the ITS, as well as each of the low-copy nuclear genes, with topologies compatible with the inferred species relationships. For these markers, an outgroup from outside the Neurachninae was added to the data set and used to estimate the first divergence time of the Neurachninae, but was removed during the molecular dating analyses.

Results

Photosynthetic pathway variation in the Neurachninae

Leaf δ$_{13}$C assays confirmed previous photosynthetic pathway determinations in the Neurachninae (Hattersley and Roksandic, 1983; Hattersley et al., 1986; Macfarlane, 2007). Mean δ$_{13}$C values near –13% in N. munroi and P. muelleri demonstrate that these species are C$_4$ (Table 1). All other Neurachninae members, including the C$_3$-C$_4$ intermediate N. minor, showed δ$_{13}$C values between –24‰ and –29‰, which are typical of C$_3$ plants (Table 1).

Neurachninae genome size analyses

The 2C DNA estimates varied 4-fold from 0.98 ± 0.01 pg DNA for the diploid N. lanigera S.T.Blake to 3.97 ± 0.04 pg DNA for the tetraploid P. muelleri (Table 1). These values are well within the range observed in the Poaceae (Bennett and Leitch, 2010, Plant DNA C-values database, http://data.kew.org/evalues/), and agree with previous estimates from the Neurachninae based on 2C values or chromosome counts (Prendergast and Hattersley, 1985; Morgan and Westoby, 2005; Macfarlane, 2007).

Intraspecific ploidy variation was found in N. alopecuroidea R.Br. (4x, 5x, 6x, and 7x), and although multiple ploidy levels have been observed previously for N. lanigera and N. munroi (Prendergast and Hattersley, 1985), only diploid and tetraploid forms of these species, respectively, were detected in this study.

Phylogenetic relationships among the Neurachninae

All plastid and nuclear markers showed that Neurachne and Paraneurachne species formed a strongly supported monophyletic
clade, which was sister to *Thyridolepis* spp., and the monospecific *Paraneurachne* was nested inside *Neurachne* (Supplementary Figs S1–S4 at JXB online). The original name of this species, *Neurachne muelleri* Hack. (Hackel, 1895), should thus be resurrected.

Based on plastid markers, each species of the *Neurachne/Paraneurachne* group was strongly supported as monophyletic, with the exception of *N. lanigera*, which was poorly resolved with respect to *N. alopecuroidea* (Supplementary Fig. S1 at JXB online). The two C₄ taxa (*N. munroi* and *P. muelleri*) were not closely related, and the C₃–C₄ intermediate *N. minor* was not sister to either of the C₄ taxa (Supplementary Fig. S1).

The topology of the phylogeny inferred from ITS (Supplementary Fig. S2 at JXB online) was almost identical to that from plastid markers. The only exception was *N. queenslandica* S.T.Blake, which was positioned as sister to *N. alopecuroidea* and not *P. muelleri*. Forcing *N. queenslandica* to be sister to *N. alopecuroidea* in the phylogeny inferred from plastid markers led to a significant decrease of likelihood (S-H test, difference of log-likelihoods = –2.786, $P < 0.005$). This incongruence between the plastid and ITS inferred phylogenies thus appears real and is symptomatic of incomplete lineage sorting or reticulate evolution. Since *N. queenslandica* is hexaploid (Prendergast and Hattersley, 1985), an allopolyploid origin is likely, with the female parent related to the *P. muelleri* lineage and the male parent to the *N. alopecuroidea/N. lanigera* lineage (assuming maternal chloroplast inheritance). *Neurachne queenslandica*, therefore, may be a natural hybrid between C₃ and C₄ parents, although further support for this hypothesis is required as C₄ photosynthesis may have evolved in *P. muelleri* after the hybridization event.

The phylogeny inferred from combined plastid and ITS markers (plastid+ITS) was strongly resolved (Fig. 1), and was thus considered representative of the species relationships. In this phylogeny, the monophyly of all species was strongly supported, with the exception of *Thyridolepis* taxa.

The relationships between species inferred from *aroodeh* (Supplementary Fig. S3 at JXB online) and *waxy* (Supplementary Fig. S4) were poorly supported, but nevertheless compatible with the plastid+ITS phylogeny. Forcing the topologies to that deduced from plastid+ITS did not significantly decrease the likelihood (S-H test, difference of log-likelihoods = –2.786 and –0.822, respectively, $P = 0.257$ and 0.269, respectively). All *aroodeh* and *waxy* sequences isolated from the same species were monophyletic, with the exception of the sequences encoding these markers isolated from *T. multiculmis* S.T.Blake. Two sequences encoding both *waxy* and *aroodeh* were isolated from this species, and in both cases one sequence was more similar to that from *T. multiculmis* S.T.Blake than it was to the other sequence isolated from *T. multiculmis*.

### Evolutionary history of ppc-B2 in the Neurachninne

The coding sequences encompassing exons 5–10 (1786 bp; 270 parsimoniously informative sites within the Neurachninne) of the Neurachninne *ppc-B2* genes were placed in a larger data set

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**Table 1.** Carbon isotope ratios ($\delta^{13}$C) and genome size data for Neurachninne species. Carbon isotope ratios are average values ± standard deviation (SD) determined from multiple herbarium samples of the same species (Supplementary Appendix S1 at JXB online). Photosynthetic pathway designations are shown based on these results and Hattersley et al. (1986) for the C₃–C₄ species *N. minor*. Genome size measurements ± SD were calculated from the average genome sizes of individual homoploids, which were measured from fresh leaf material on three consecutive days (see the Materials and Methods for further detail).

<table>
<thead>
<tr>
<th>Species</th>
<th>$\delta^{13}$C ± SD</th>
<th>2C ± SD (pg)</th>
<th>2C (Mbp)</th>
<th>1Cx ± SD (pg)</th>
<th>2n</th>
<th>Ploidy</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Neurachne alopecuroidea</em></td>
<td>–27.1 ± 1.5</td>
<td>2.24 ± 0.02</td>
<td>2190</td>
<td>0.56 ± 0.01</td>
<td>36</td>
<td>4x</td>
</tr>
<tr>
<td><em>N. annularis</em></td>
<td>–24.8 ± 0.0</td>
<td>1.30 ± 0.01</td>
<td>1270</td>
<td>0.65 ± 0.01</td>
<td>18</td>
<td>2x</td>
</tr>
<tr>
<td><em>N. lanigera</em></td>
<td>–28.4 ± 1.2</td>
<td>0.98 ± 0.01</td>
<td>958</td>
<td>0.49 ± 0.01</td>
<td>18</td>
<td>2x</td>
</tr>
<tr>
<td><em>N. minor</em> (<em>C₃–C₄</em>)</td>
<td>–27.1 ± 1.0</td>
<td>2.76</td>
<td>2700</td>
<td>0.69</td>
<td>36</td>
<td>4x</td>
</tr>
<tr>
<td><em>N. munroi</em> (<em>C₃–C₄</em>)</td>
<td>–12.7 ± 1.0</td>
<td>3.61</td>
<td>3530</td>
<td>0.60</td>
<td>36</td>
<td>4x</td>
</tr>
<tr>
<td><em>N. queenslandica</em></td>
<td>–27.1 ± 0.4</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>54</td>
<td>6x</td>
</tr>
<tr>
<td><em>N. terufolia</em></td>
<td>–25.7 ± 0.5</td>
<td>1.48</td>
<td>1450</td>
<td>0.74</td>
<td>18</td>
<td>2x</td>
</tr>
<tr>
<td><em>Paraneurachne muelleri</em></td>
<td>–13.3 ± 0.4</td>
<td>3.97 ± 0.03</td>
<td>3880</td>
<td>0.99 ± 0.01</td>
<td>36</td>
<td>4x</td>
</tr>
<tr>
<td><em>Thyridolepis mitchelliana</em></td>
<td>–26.6 ± 1.4</td>
<td>1.32 ± 0.01</td>
<td>1290</td>
<td>0.66 ± 0.01</td>
<td>18</td>
<td>2x</td>
</tr>
<tr>
<td><em>T. multiculmis</em></td>
<td>–26.3 ± 1.8</td>
<td>2.50 ± 0.02</td>
<td>2450</td>
<td>0.63 ± 0.01</td>
<td>36</td>
<td>4x</td>
</tr>
<tr>
<td><em>T. xerophila</em></td>
<td>–27.6 ± 0.6</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>18</td>
<td>2x</td>
</tr>
</tbody>
</table>

ND, not determined.

* a Ploidy and chromosome numbers are inferred from the flow cytometry results of this study; other values are from Prendergast and Hattersley (1985) and Macfarlane (2007).
Multiple photosynthetic transitions in the Neurachninae | 6301

encompassing many Panicoideae ppc-B2 sequences (Christin et al., 2012b). In this inferred phylogeny, the Neurachninae sequences were positioned near those of affiliated C3 Paniceae, and were monophyletic, with the exception of one Ancistrachne sequence, which was positioned outside the Neurachninae and was therefore excluded from further analyses.

Inside the Neurachninae, despite a lack of resolution of the deeper nodes, the phylogenetic relationships deduced from ppc-B2 sequences were almost identical to those deduced from other markers (Supplementary Fig. S5 at JXB online). The only exceptions were the sequences from P. muelleri and N. munroi. Three groups of highly divergent ppc-B2 sequences were retrieved from P. muelleri. One of these groups was supported as sister to N. alopecuroidea/N. lanigera ppc-B2 sequences, as expected based on other markers (Fig. 1). The exact positions of the other two clusters of P. muelleri ppc-B2 sequences, however, were not resolved with confidence, although one of them is strongly supported as sister to sequences from N. munroi (Supplementary Fig. S5, subclade highlighted in red). The close relationship between N. munroi ppc-B2 sequences and some P. muelleri ppc-B2 sequences was strongly supported even when a phylogeny was inferred from the five introns only (774 aligned base pairs, including 129 parsimoniously informative sites within the Neurachninae; Supplementary Fig. S6, subclade highlighted in red).

Different hypotheses reconciling ppc-B2 phylogeny with those inferred from other markers were evaluated using the sequence data (Fig. 2). Forcing the monophyly of all P. muelleri ppc-B2 sequences and placing them as expected for the species, and placing the N. munroi ppc-B2 sequences in the predicted species position—the hypothesis of gene transmission that followed species genealogy—very strongly decreased the likelihood of the data (S-H test, difference of log-likelihoods= –246.516, P < 0.0001). Thus the close relationship between some of the P. muelleri ppc-B2 sequences and the N. munroi ppc-B2 sequences is unambiguous. A scenario in which a gene transfer occurred from N. munroi to P. muelleri involved placing the closely related N. munroi and P. muelleri sequences as expected for N. munroi, and the other P. muelleri sequences as expected for this species (Fig. 2A). This treatment of the data did not significantly affect the likelihood (S-H test, difference of log-likelihoods= –5.542, P=0.621). The alternative hypothesis, a gene transfer from P. muelleri to N. munroi, was also examined, and involved moving all N. munroi and P. muelleri ppc-B2 sequences to the position expected for P. muelleri (Fig. 2B). Again, no significant effect on the likelihood was seen (S-H test, difference of log-likelihoods= –6.799, P=0.588). The hypothesis that the data set might encompass different paralogues that appeared through gene duplication before the diversification of Neurachne/Paraneurachne was also tested. This implied placing P. muelleri sequences as sister to all other Paraneurachne/Neurachne sequences (Fig. 2C), which did not significantly decrease the likelihood (S-H test, difference of log-likelihoods= –9.391, P=0.485). This hypothesis, however, is not supported by the molecular dating analyses, which indicate that

![Fig. 1. Phylogeny of the Neurachninae, photosynthetic types, and ploidy levels. The tree was obtained through Bayesian inference using the plastid markers ndhF, trnK/matK, trnLF, rpl16, and rpoC2, and the genomic region encoding the internal transcribed spacer 1, 5.8S rRNA, and internal transcribed spacer 2 (ITS). Bayesian support values and bootstrap values are indicated above and below branches, respectively. The tree was calibrated and branch lengths are proportional to divergence times, in million years ago (Ma). Photosynthetic types are indicated on the right, as are ploidy levels. Multiple branches to the same species denote different genes or alleles.](https://academic.oup.com/jxb/article-abstract/63/17/6297/564800)
the divergence of the closely related *N. munroi* and *P. muelleri* ppc-B2 genes occurred long after the initial diversification of *Neurachne/Paraneurachne* (Fig. 2C). Finally, it was considered whether the phylogenetic analyses might be biased by convergent amino acid changes in these two *C₄* species (Christin et al., 2007, 2012a). However, topological tests based only on introns yielded identical results, ruling out such a scenario. The data, therefore, show that at least some Neurachninae ppc-B2 genes were not transmitted following the species genealogy, but the direction of transmission, *N. munroi* to *P. muelleri* or vice versa, cannot be determined with confidence from the current data.

Residues at positions putatively selected for *C₄*-specific function (Christin et al., 2007) were compared among the predicted amino acid sequences of the Neurachninae ppc-B2 sequences (Fig. 3). Of the 12 positions with the highest probability of having been positively selected in grasses (Christin et al., 2007), four were conserved in all members of the Neurachninae, with amino acid residues characteristic of non-*C₄* ppc-B2 proteins (positions 577, 637, 761, and 807). At the other positions, the predicted amino acid sequences encoded by the closely related *N. munroi* and *P. muelleri* ppc-B2 genes presented residues that characterize independently evolved *C₄* ppc-B2 genes (Fig. 3), with a serine at position 780 that was shown to be a major determinant of *C₄*-specific PEPC activity (Błasing et al., 2000; Svensson et al., 2003). The other *P. muelleri* ppc-B2 sequences were predicted to encode some *C₄*-characteristic residues (Fig. 3), but also contained several stop codons and deletions altering the reading frame, and are very probably non-functional pseudogenes.
Multiple photosynthetic transitions in the Neurachninae

Molecular dating of the Neurachninae

The confidence intervals of estimated species divergence times based on plastid and nuclear markers overlapped (Fig. 4). Within most species, the divergence time between nuclear sequences overlaps with the crown age of the species. However, N. munroi arodeh sequences are estimated to have diverged long before the different N. munroi accessions. The occurrence of this pattern on a single gene can be explained by gene duplication or polyploidy followed by the loss of some gene copies. In addition, with multiple nuclear markers, the divergence of the different T. multiculmis sequences is estimated before the divergence of the different Thyridolepis species. This pattern, restricted to the tetraploid T. multiculmis, suggests an allopolyploid origin, with parents inside Thyridolepis, one of which was either not sampled or is extinct. The different hypotheses for the evolutionary history of ppc-B2 described above yielded similar age estimates (Fig. 2).

Discussion

Evolutionary origins of C₄ biochemistry in the Neurachninae

The C₃ and C₃–C₄ members of the Neurachninae are not sister species, but are separated in both plastid- and nuclear marker-based phylogenies by C₃ taxa (Fig. 1; Supplementary Figs S1–S4 at JXB online). In other groups containing species demonstrating different photosynthetic types, and for which robust phylogenies exist, some C₃–C₄ species are directly sister to C₄ taxa as in Flaveria (McKown et al., 2005), Cleome (Feodorova et al., 2010), Heliotropium (Hilger and Diane, 2003), and the Molluginaceae (Christin et al., 2011). This pattern suggests that the photosynthetic variation observed in extant taxa reflects a gradual transition from C₃ to C₃–C₄ and finally to C₄ photosynthesis. However, several C₃–C₄ taxa are not closely related to any C₄ species (Sage et al., 2011), and many groups encompass a mixture of C₃, C₃–C₄, and C₄ taxa, including the Neurachninae, but also other Flaveria (McKown et al., 2005) and Molluginaceae (Christin et al., 2011) lineages, sedges (Roalson et al., 2010), and the Chenopodiaceae (Sage et al., 2011).

The clustering of taxa using different photosynthetic pathways that is observed in the phylogenetic trees of the Neurachninae and other groups suggests a complex evolutionary history, with either multiple transitions from C₃ to C₃–C₄ or C₄ photosynthesis, multiple losses of an ancestral C₄ or C₃–C₄ type, or a combination of both (Christin et al., 2010). A simple scenario of a single C₄ origin followed by reversals to the C₃ (or C₃–C₄) state can be ruled out for the Neurachninae by the analyses of genes encoding PEPC. During the evolution of this key C₄ pathway enzyme, mutations occurred in the genes that resulted in modifications to the catalytic and regulatory properties of the encoded proteins, allowing them to function optimally in C₄ photosynthesis (Dong et al., 1998; Bläsing et al., 2000). These mutations often occur at identical positions in the PEPC coding sequences of distantly related species (Christin et al., 2007; Besnard et al., 2009). Several amino acid changes characteristic of C₄-specific PEPC were predicted from the ppc-B2 genes of C₄.
Neurachninae, including the key $C_4$ determinant alanine to serine mutation at position 780 (Fig. 3). If the $C_4$-specific properties of PEPC appeared before the diversification of the Neurachninae, the $ppc-B2$ genes of non-$C_4$ taxa most probably would have maintained codons for some $C_4$-characteristic amino acids. However, the $ppc-B2$ genes of the $C_3$ and $C_3–C_4$ Neurachninae do not encode $C_4$-specific amino acids, and are indistinguishable at these positions from species known never to have been in a $C_4$ state (Fig. 3), strongly suggesting that the $ppc-B2$ genes of these non-$C_4$ taxa did not descend from genes encoding enzymes that were $C_4$ optimized.

The $C_4$-specific properties of the PEPC used by $N. muenroi$ and $P. muelleri$ evolved after the divergence from non-$C_4$ Neurachninae, but the $C_4$ PEPC genes of these two species strongly grouped together in the phylogeny, even when only introns were considered, arguing against genetic convergence as a phylogenetic bias (Supplementary Figs S5, S6 at JXB online). The divergence of these genes occurred long after the split of

Fig. 4. Divergence times for members of the Neurachninae estimated from different markers. The inferred chronograms are represented for (A) plastid markers, (B) ITS; (C) arodeh, and (D) waxy. Branch lengths are proportional to divergence times, in million years ago (Ma). For the plastid markers, the main taxa are compressed. For nuclear markers, sequences with low divergence and belonging to the same species are compressed. Confidence intervals of age estimates are represented by grey boxes for interspecific nodes in all trees, and species divergence in the plastid tree only.
N. munroi and the lineage leading to P. muelleri (Fig. 2), excluding paralogy as an explanation for this grouping. Therefore, the transmission of these C₄-optimized genes did not follow the species genealogy. A lateral transfer might have occurred via allopolyploidization, but this is unlikely given that none of the other nuclear markers isolated from P. muelleri showed affinities with those of N. munroi. It is more likely that the transmission occurred via hybridization, followed by backcrossing and introgression into the population, or alternatively through a lateral gene transfer event, as shown for the grass Allotropis (Christin et al., 2012h), and suggested for the sedge Eleocharis (Besnard et al., 2009). Although the direction of the ppc-B2 gene transfer is not known yet, a transfer from N. munroi to P. muelleri is more consistent with the present data. In some regions of Australia, N. munroi and P. muelleri co-occur (Prendergast and Hattersley, 1985; Supplementary Fig. S7), providing opportunities for a gene transfer that might have been favoured by natural selection. Some of the native P. muelleri ppc-B2 copies encode residues at positions 466 and 531 that generally characterize C₄-specific PEPC (Besnard et al., 2009), which suggests that these genes previously fulfilled the C₄ function in P. muelleri and underwent a few changes toward C₃ optimization of the encoded enzyme. The acquisition of ppc-B2 genes from N. munroi that were more C₄ optimized would have been advantageous, leading to a replacement of the vertically acquired P. muelleri genes, and their subsequent pseudogenization (Fig. 3).

Anatomical enablers of photosynthetic transitions in the Neurachninae

The optimization of C₄ biochemistry is hypothesized to be one of the final steps in the evolution of C₄ plants (Sage et al., 2012), with the initial steps involving the reduction of the mesophyll cell to bundle sheath cell ratio, the enhancement of bundle sheath organelles, and the appearance of a photosynthetic CO₂ pump, which characterizes nearly all known C₃-C₄ intermediates (Hylton et al., 1988; Griffiths, 1989; Hattersley and Watson, 1992; Sage et al., 2012). The C₄ P. muelleri and N. munroi and the C₃-C₄ N. minor have very similar foliar anatomies, with short interveinal distances, low mesophyll to bundle sheath tissue volume, and high numbers of bundle sheath organelles, typical of C₄ taxa (Hattersley et al., 1982, 1986; Brown and Hattersley, 1989). The C₃ taxa that separate them in the phylogeny have a comparatively larger interveinal distance and smaller bundle sheath cells (Hattersley et al., 1982, 1986; Macfarlane, 2007), although the interveinal distance tends to be shorter than that of typical C₃ taxa in N. queenslandica and N. annularis Macfarlane, and especially in N. tenuifolia S.T.Blake, where additional minor bundles reduce the average interveinal distance (Hattersley et al., 1982; Macfarlane, 2007). C₃ species in the Neurachne/Thryidolepis clade have conspicuous mestome sheaths, the tissue that has been recruited for carbon reduction in C₃-C₄ and C₄ Neurachninae species (Dengler et al., 1985). Indeed, while this tissue is usually devoid of organelles in C₃ taxa, it contains some chloroplasts and a high number of mitochondria in these C₃ Neurachninae (Hattersley et al., 1982, 1986; Hattersley and Roksandic, 1983). The presence of these organelles in the mestome sheath may have facilitated the evolution of a photorespiratory CO₂ pump in the Neurachninae, enabling the subsequent transition to a complete C₄ pathway (Sage et al., 2012). As has been hypothesized for other anatomical characters in some eudicot lineages with multiple C₄ origins (Marshall et al., 2007; McKown and Dengler, 2007; Christin et al., 2011), the high organelle content of C₃ Neurachninae mestome sheaths may have contributed to the multiple origins of biochemical carbon-concentrating mechanisms in this grass group.

Recurrent polyploidizations in the Neurachninae as diversification facilitators?

The distribution of ploidy levels in the species phylogeny (Fig. 1) suggests either multiple polyploidy events or a few followed by a return to a diploid state (diploidization). The latter suggestion can be disregarded because it would have led to a high chromosome number in the newly formed diploids, which is not observed in the Neurachninae (Table 1). In addition, multiple polyploidizations are supported by the existence of intraspecific ploidy variation in several taxa (Table 1; Prendergast and Hattersley, 1985). The data therefore suggest that seven independent polyploidization events have occurred in the Neurachne/Thryidolepis clade, one per polyploid species (Fig. 1).

The analyses of markers from multiple genomes identified T. multicalmis and N. queenslandica as probable allotetraploids, the latter possibly representing a hybrid from a C₃ and a C₄ parent. In contrast, the tetraploid C₃-C₄ intermediate N. minor does not appear to be the result of an allopolyploidization between C₃ and C₄ Neurachninae species, or at least not between known members, as the sequences from N. minor clustered together in the phylogenies at the same position for all plastid and nuclear markers (Fig. 1; Supplementary Figs S1–S4 at JXB online). With leaf anatomy similar to that of the C₄ N. munroi (Hattersley et al., 1982), and a typically C₃ isotopic value (Table 1; Hattersley and Roksandic, 1983; Hattersley et al., 1986), consistent with minimal or limited C₃ cycle activity (Hattersley and Stone, 1986; Hattersley et al., 1986; von Caemmerer, 1992), N. minor may represent a transitional stage toward an additional origin of full C₄ photosynthesis in the Neurachninae. As for N. minor, all the plastid and nuclear marker sequences belonging to the polyploid N. alopecuroidea, N. lanigera, and N. munroi clustered together in the phylogenies (Fig. 1; Supplementary Figs S1–S6), suggesting that the dominant mechanism for increased chromosome numbers in this grass group was autopolyploidization.

The two C₄ taxa and the C₃-C₄ intermediate species of the Neurachninae are all polyploids of putatively recent origin (Fig. 1), and autopolyploidization might have allowed photosynthetic diversification by providing additional copies of genes encoding enzymes that fulfilled housekeeping functions in the C₃ ancestors. A relaxation of selection due to genetic redundancy has indeed been suggested to facilitate the recruitment of enzymes into novel photosynthetic functions (Monson, 2003; Wang et al., 2009); however, as yet no empirical evidence exists to support this hypothesis, and comparison of distantly related grass genomes questioned the importance of gene duplication in the evolution of the C₄ syndrome (Williams et al., 2012). The apparent correlation of autopolyploidy and derived photosynthetic types in the Neurachninae offers an opportunity to test the
link between genetic redundancy and photosynthetic diversification within a sound phylogenetic framework.

Conclusion

The Neurachninae have long been viewed as a potential model for understanding the evolution of C₄ photosynthesis in the grasses due to natural photosynthetic variation in the group; however, evolutionary inferences were hampered by the lack of a phylogenetic framework. Using multiple markers from the chloroplast and nuclear genomes, it has been shown that C₄ biochemistry has evolved more than once in this grass group, and may have been facilitated by anatomical enablers, a high frequency of autopolyploidization, and transfer of C₄-optimized genes between related taxa. Molecular dating indicates that these events most probably occurred during the late Pliocene, an epoch of relatively low atmospheric CO₂ (Gerhart and Ward, 2010), when the Australian continent became drier (Bowler, 1976). The present-day distribution of extant Neurachninae (Prendergast and Hattersley, 1985) indicates that the group appeared in the red sand soils and stony rises of semi-arid west-central Australia. These observations are consistent with recent models of C₄ evolution that hypothesize that climate deterioration in the past 5 million years selected for a series of traits that predisposed taxa to evolve novel photosynthetic mechanisms to deal with the combined effects of severe stress and low atmospheric CO₂ (Sage et al., 2012).

Supplementary data

Supplementary data are available at JXB online.

Figure S1. Neurachninae phylogeny inferred from plastid markers.

Figure S2. Neurachninae phylogeny inferred from the ITS region.

Figure S3. Phylogenetic relationships among arodeh sequences.

Figure S4. Phylogenetic relationships among waxy sequences.

Figure S5. Phylogenetic relationships among ppc-B2 sequences.

Figure S6. Phylogenetic relationships among ppc-B2 sequences deduced from intron sequences.

Figure S7. Distribution of Neurachne spp. and Paraneurachne muelleri in Australia.

Table S1. Neurachne, Paraneurachne, and Thyridolepis samples used for genome size analyses.

Table S2. Plastid and ITS sequences from Neurachne, Paraneurachne, and Thyridolepis species used in phylogenetic analyses.

Table S3. Primers used in this study.

Table S4. Neurachninae sampled for low-copy number nuclear markers.

Appendix S1. Herbarium vouchers for specimens of Neurachne, Paraneurachne, and Thyridolepis sampled for carbon isotope assays.

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References


Multiple photosynthetic transitions in the Neurachininae | 6307


