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RESEARCH PAPER

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 C_3, C_4, and C_3–C_4 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 C_4 photosynthesis. Phylogenetic analyses also showed that following their separate C_4 origins, these two taxa exchanged a gene encoding the C_4 form of phospho*enol*pyruvate carboxylase. The C_3–C_4 intermediate *Neurachne minor* S.T.Blake is phylogenetically distinct from the two C_4 lineages, indicating that intermediacy in this species evolved separately from transitional stages preceding C_4 origins. The Neurachninae shows a substantial capacity to evolve new photosynthetic pathways repeatedly. Enablers of these transitions might include anatomical pre-conditions in the C_3 ancestor, and frequent autopolyploidization. Transfer of key C_4 genetic elements between independently evolved C_4 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: C_4 grass evolution, C_4 photosynthesis, C_3–C_4 intermediate, grass phylogeny, lateral gene transfer, *Neurachne*, *Paraneurachne*, polyploidy

Introduction

Despite its complexity, the C_4 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 C_4 lineages have been postulated in the PACMAD clade (Grass Phylogeny Working Group II, 2012). Such a clustering of C_4 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., 1997; 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; Muhaidat 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 Neurachninae; Morrone et al., 2012), a group of three genera and 11 species endemic to Australia (Blake, 1972; Macfarlane, 2007).

Within the Neurachninae, 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 (*Thyridolepsis*, *Ancistrachne*, *Cleistochloa*, and *Calyptochloa*) are C_3. The clade belongs to the Panicoideae 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 Neurachninae 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 Neurachninae, 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 Neurachninae, 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 Neurachninae species, and revealed the genome dynamics of the group. The outcomes also highlight the diversity of photosynthetic transitions in the Neurachninae, 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 Neurachninae 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 Neurachninae species were also sampled for phylogenetic analyses; these were herbarium specimens or plants collected from the field (Supplementary Tables S1, S2).

Carbon isotope ratios ($\delta^{13}C$)

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://stableisotopefacility.ucdavis.edu). Some of the samples were previously assayed for $\delta^{13}C$ (Hattersley and Roksandic, 1983; Hattersley et al., 1986).

Determination of genome size

Nuclei were simultaneously released from fresh leaf material from a Neurachninae species and a calibration standard [*Raphanus sativus* cv. Saxa (2C DNA content = 1.11 pg; Doležel et al., 1992) or *Lepidosperma 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 cytometer (Becton Dickinson, Franklin Lakes, NJ, USA) with 488 nm excitation and 585/424 band pass filter detection. Data were collected with BD FACSDIVA 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 homoploids (Supplementary Table S1 at *JXB* online) were calculated using the average genome sizes of the individual homoploids. 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 *trnLF*) that have been used previously to investigate relationships among grasses

(Duvall *et al*., 2003; Salariato *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 *arodeh* were amplified with previously designed primers (Christin *et al*., 2012*b*). The *ppc-B2* lineage of phospho*enol*pyruvate 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, 2012*b*). These three markers were isolated as described previously (Christin *et al*., 2012*b*), 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*., 2012*b*; 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 trees were obtained by Bayesian inference with MrBayes 3.2 (Ronquist and Huelsenbeck, 2003). Two analyses, each consisting of four parallel chains, were run for 7 000 000 generations after a burn-in period of 3 000 000, sampling a tree each 1000 generations. A consensus tree was computed on the 14 000 sampled trees. Phylogenetic trees were also inferred under a maximum likelihood (ML) criterion with the software PhyML (Guindon and Gascuel, 2003). Branch support of the ML trees was evaluated with 100 bootstrap pseudo-replications.

Phylogenetic trees were inferred for concatenated plastid data and ITS separately, and a further estimate of phylogeny was carried out by concatenating these two data sets. The three low-copy nuclear markers were analysed separately. For *waxy*, parts of the sequences that consisted of simple sequence repeats or short tandem repeats were difficult to align and were removed from the data set, resulting in the elimination of 52 aligned base pairs. To decrease the possibility of a bias due to convergent evolution (Christin *et al*., 2007, 2012*a*), *ppc-B2* phylogeny was also inferred from introns only. The significance of topological incongruence between the different markers was evaluated by Shimodaira–Hasegawa (S-H) tests with multiple-comparison correction (Shimodaira and Hasegawa, 1999), as implemented in baseml software (Yang, 2007).

Molecular dating

The divergence times of species within the Neurachninae were estimated with a molecular dating approach, which allows for rate variation among branches, following the recommendations from Rutschmann (2006), as described in Christin *et al*. (2008). Molecular dating requires time constraints set *a priori* on some nodes of the phylogeny as minimum or maximum ages. In the absence of a reliable fossil record for this group, the root of the Neurachninae was fixed to 11 Million years ago (Ma), a preliminary estimate obtained with a larger phylogeny, and without taking phytolith fossils into account (P.-A. Christin *et al*., unpublished). If phytoliths are taken into account, this node would probably be moved to ~20 Ma (P.-A.Christin *et al*., unpublished); however, as the same constraint was set for all markers, the relative ages can be compared among the different markers.

The topology inferred from combined plastid markers and ITS was used for molecular dating as it was better resolved and more complete than those of individual markers. However, the estimation of ages was based only on plastid markers to avoid strong variation in evolutionary rates due to differences between plastid and nuclear markers. Additional molecular dating 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 $\delta^{13}C$ assays confirmed previous photosynthetic pathway determinations in the Neurachninae (Hattersley and Roksandic, 1983; Hattersley *et al*., 1986; Macfarlane, 2007). Mean $\delta^{13}C$ 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 $\delta^{13}C$ 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/cvalues/), 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. (4*x*, 5*x*, 6*x*, and 7*x*), 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

Table 1. *Carbon isotope ratios ($\delta^{13}C$) and genome size data for Neurachninae 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 C_3–C_4 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).

Species	$\delta^{13}C$ ±SD	2C ±SD (pg)	2C (Mbp)	1Cx ±SD (pg)	2*n*	Ploidy
Neurachne alopecuroidea (C_3)	–27.1 ± 1.5	2.24 ± 0.02	2190	0.56 ± 0.01	36	4*x*
		2.77 ± 0.05	2710	0.55 ± 0.01	45[a]	5*x*[a]
		3.33 ± 0.04	3260	0.55 ± 0.01	54	6*x*
		3.81	3730	0.54	63[a]	7*x*[a]
N. annularis (C_3)	–24.8 ± 0	1.30 ± 0.01	1270	0.65 ± 0.01	18	2*x*
N. lanigera (C_3)	–28.4 ± 1.2	0.98 ± 0.01	958	0.49 ± 0.01	18	2*x*
		ND	ND	ND	36	4*x*
N. minor (C_3–C_4)	–27.1 ± 1.0	2.76	2700	0.69	36	4*x*
N. munroi (C_4)	–12.7 ± 1.0	ND	ND	ND	18	2*x*
		3.61	3530	0.60	36	4*x*
		ND	ND	ND	54	6*x*
N. queenslandica (C_3)	–27.1 ± 0.4	ND	ND	ND	54	6*x*
N. tenuifolia (C_3)	–25.7 ± 0.5	1.48	1450	0.74	18	2*x*
Paraneurachne muelleri (C_4)	–13.3 ± 0.4	3.97 ± 0.03	3880	0.99 ± 0.01	36	4*x*
Thyridolepis mitchelliana (C_3)	–26.6 ± 1.4	1.32 ± 0.01	1290	0.66 ± 0.01	18	2*x*
T. multiculmis (C_3)	–26.3 ± 1.8	2.50 ± 0.02	2450	0.63 ± 0.01	36	4*x*
T. xerophila (C_3)	–27.6 ± 0.6	ND	ND	ND	18	2*x*

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).

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 C_4 taxa (*N. munroi* and *P. muelleri*) were not closely related, and the C_3–C_4 intermediate *N. minor* was not sister to either of the C_4 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= –55.801, $P < 0.001$), as did forcing it to be sister to *P. muelleri* in the ITS phylogeny (S-H test, difference of log-likelihoods= –35.332, $P < 0.005$). 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 C_3 and C_4 parents, although further support for this hypothesis is required as C_4 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 *arodeh* (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 *arodeh* 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 *arodeh* were isolated from this species, and in both cases one sequence was more similar to that from *T. mitchelliana* S.T.Blake than it was to the other sequence isolated from *T. multiculmis*.

Evolutionary history of ppc-B2 *in the Neurachninae*

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

encompassing many Panicoideae *ppc-B2* sequences (Christin *et al*., 2012*b*). In this inferred phylogeny, the Neurachninae sequences were positioned near those of affiliated C_3 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 *ppc-B2* sequences from *P. muelleri* 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* 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

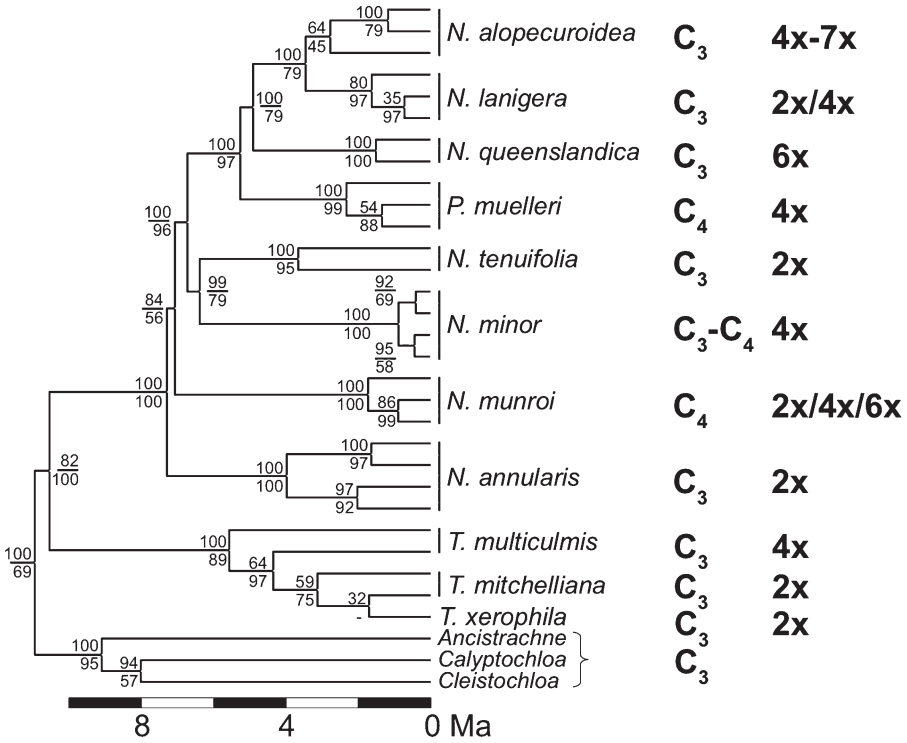

Fig. 1. Phylogeny of the Neurachninae, photosynthetic types, and ploidy levels. The tree was obtained through Bayesian inference using the plastid markers *ndhF*, *trnK/matK*, *trnLF*, *rpl16*, and *rpoC2*, and the genomic region encoding the internal transcribed spacer 1, 5.8S rRNA, and internal transcribed 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.

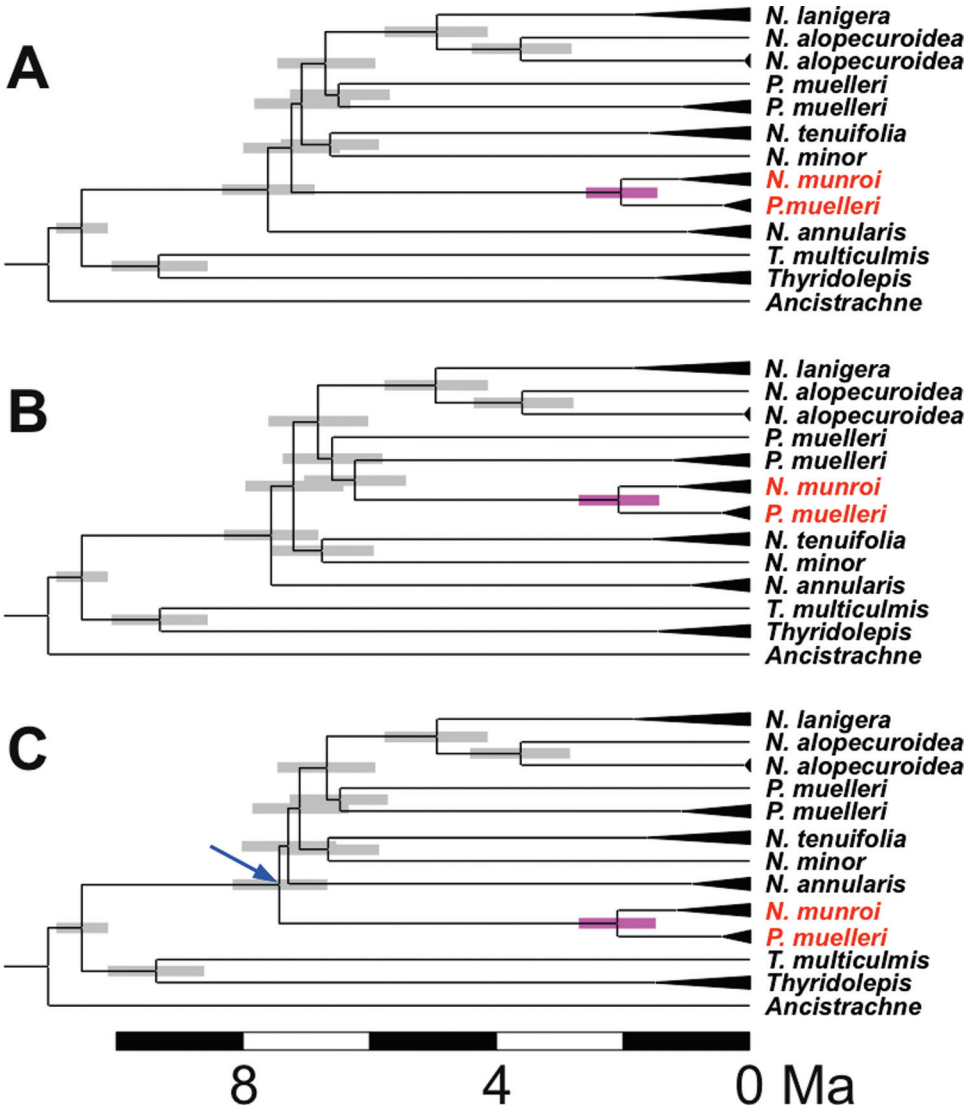

Fig. 2. Different evolutionary scenarios for *ppc-B2*, and divergence times within the Neurachninae. The inferred chronograms are represented for the different statistically plausible hypotheses: (A) gene transfer from *N. munroi* to *P. muelleri*; (B) gene transfer from *P. muelleri* to *N. munroi*; and (C) ancient duplication resulting in different paralogues being compared. Branch lengths are proportional to divergence times, in million years ago (Ma). Sequences with low divergence and belonging to the same species are compressed. Putative C_4-specific forms are highlighted in red. Confidence intervals of age estimates are represented by boxes for interspecific nodes. Purple boxes represent putative lateral gene transfer events. The blue arrow points to the ancient gene duplication postulated for hypothesis C.

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 C_4 species (Christin *et al*., 2007, 2012*a*). 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 C_4-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-C_4 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 C_4 *ppc-B2* genes (Fig. 3), with a serine at position 780 that was shown to be a major determinant of C_4-specific PEPC activity (Bläsing *et al*., 2000; Svensson *et al*., 2003). The other *P. muelleri ppc-B2* sequences were predicted to encode some C_4-characteristic residues (Fig. 3), but also contained several stop codons and deletions altering the reading frame, and are very probably non-functional pseudogenes.

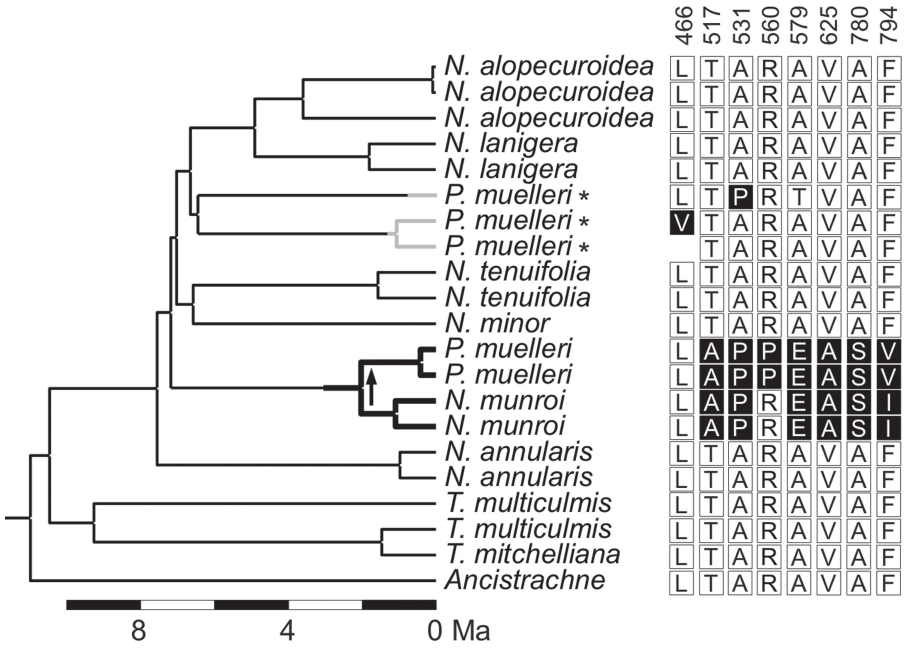

Fig. 3. Evolutionary history of *ppc-B2* in the Neurachninae. The calibrated phylogeny represents one of the hypotheses compatible with the species relationships, which assumes a gene transfer from *N. munroi* to *P. muelleri* (see Fig. 2A). Branch lengths are proportional to divergence times, in million years ago (Ma). Branches leading to the putative C_4-specific genes are shown in bold. The black arrow indicates a putative horizontal transfer. Pseudogenes are indicated by asterisks and grey branches. Amino acids at the positions hypothesized to have been under C_4-specific selection and that were variable inside the Neurachninae are shown on the right. C_4-characteristic residues are highlighted in black.

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 C_4 biochemistry in the Neurachninae

The C_4 and C_3–C_4 members of the Neurachninae are not sister species, but are separated in both plastid- and nuclear marker-based phylogenies by C_3 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 C_3–C_4 species are directly sister to C_4 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 C_3 to C_3–C_4 and finally to C_4 photosynthesis. However, several C_3–C_4 taxa are not closely related to any C_4 species (Sage *et al*., 2011), and many groups encompass a mixture of C_3, C_3–C_4, and C_4 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 C_3 to C_3–C_4 or C_4 photosynthesis, multiple losses of an ancestral C_4 or C_3–C_4 type, or a combination of both (Christin *et al*., 2010). A simple scenario of a single C_4 origin followed by reversals to the C_3 (or C_3–C_4) state can be ruled out for the Neurachninae by the analyses of genes encoding PEPC. During the evolution of this key C_4 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 C_4 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 C_4-specific PEPC were predicted from the *ppc-B2* genes of C_4

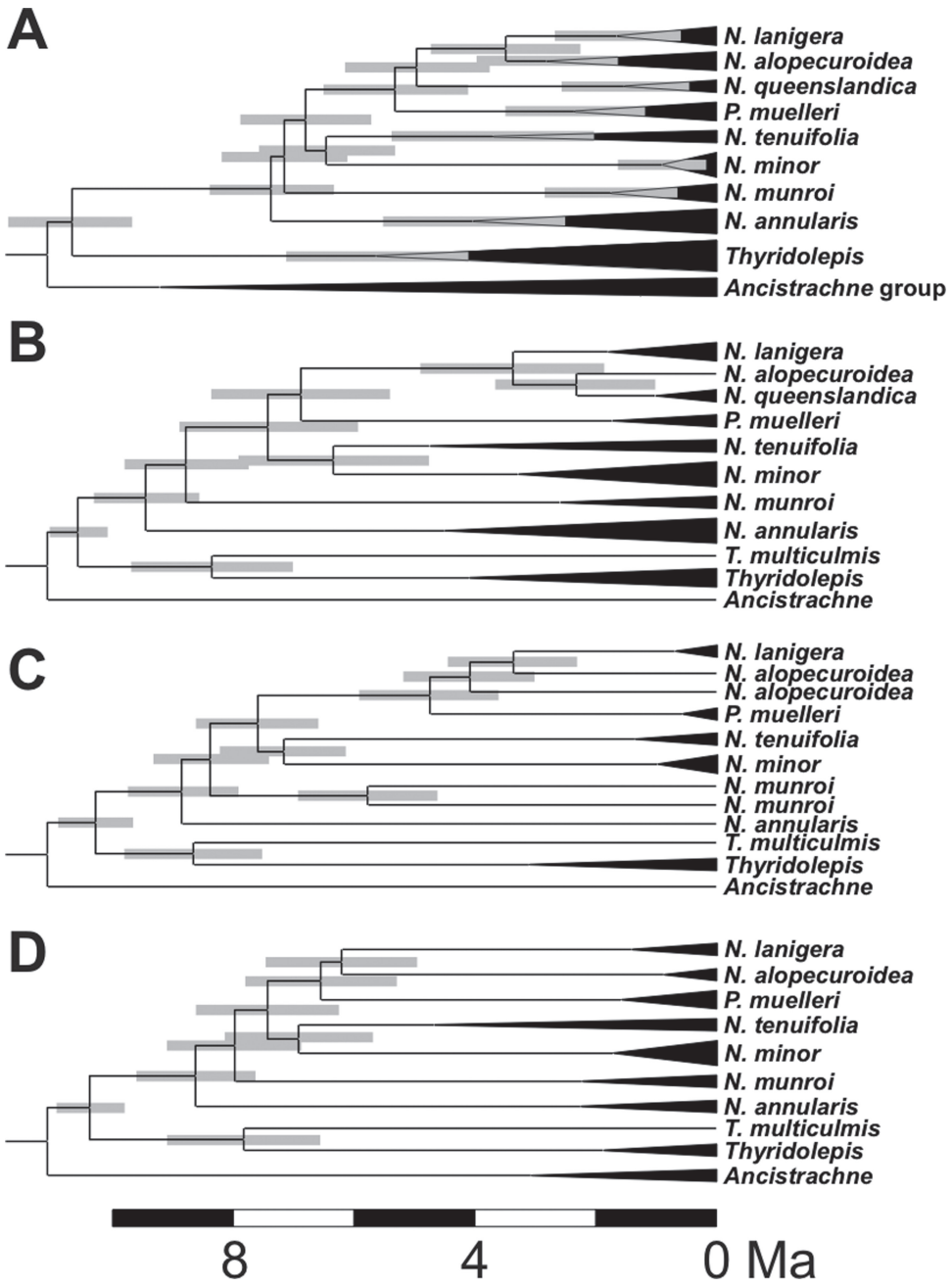

Fig. 4. Divergence times for members of the Neurachninae estimated from different markers. The inferred chronograms are represented for (A) plastid markers, (B) ITS; (C) *arodeh*, and (D) *waxy*. Branch lengths are proportional to divergence times, in million years ago (Ma). For the plastid markers, 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.

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. munroi* 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

N. munroi and the lineage leading to *P. muelleri* (Fig. 2), excluding paralogy as an explanation for this grouping. Therefore, the transmission of these C_4-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 *Alloteropsis* (Christin *et al*., 2012*b*), 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 C_4-specific PEPC (Besnard *et al*., 2009), which suggests that these genes previously fulfilled the C_4 function in *P. muelleri* and underwent a few changes toward C_4 optimization of the encoded enzyme. The acquisition of *ppc-B2* genes from *N. munroi* that were more C_4 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 C_4 biochemistry is hypothesized to be one of the final steps in the evolution of C_4 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 photorespiratory CO_2 pump, which characterizes nearly all known C_3–C_4 intermediates (Hylton *et al*., 1988; Griffiths, 1989; Hattersley and Watson, 1992; Sage *et al*., 2012). The C_4 *P. muelleri* and *N. munroi* and the C_3–C_4 *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 C_4 taxa (Hattersley *et al*., 1982, 1986; Brown and Hattersley, 1989). The C_3 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 C_3 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). C_3 species in the *Neurachne*/*Thyridolepis* clade have conspicuous mestome sheaths, the tissue that has been recruited for carbon reduction in C_3–C_4 and C_4 Neurachninae species (Dengler *et al*., 1985). Indeed, while this tissue is usually devoid of organelles in C_3 taxa, it contains some chloroplasts and a high number of mitochondria in these C_3 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 CO_2 pump in the Neurachninae, enabling the subsequent transition to a complete C_4 pathway (Sage *et al*., 2012). As has been hypothesized for other anatomical characters in some eudicot lineages with multiple C_4 origins (Marshall *et al*., 2007; McKown and Dengler, 2007; Christin *et al*., 2011), the high organelle content of C_3 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/Thyridolepis* clade, one per polyploid species (Fig. 1).

The analyses of markers from multiple genomes identified *T. multiculmis* and *N. queenslandica* as probable allotetraploids, the latter possibly representing a hybrid from a C_3 and a C_4 parent. In contrast, the tetraploid C_3–C_4 intermediate *N. minor* does not appear to be the result of an allopolyploidization between C_3 and C_4 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 C_4 *N. munroi* (Hattersley *et al*., 1982), and a typically C_3 isotopic value (Table 1; Hattersley and Roksandic, 1983; Hattersley *et al*., 1986), consistent with minimal or limited C_4 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 C_4 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 C_4 taxa and the C_3–C_4 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 C_3 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 C_4 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 C_4 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 C_4 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 C_4-optimized genes between related taxa. Molecular dating indicates that these events most probably occurred during the late Pliocene, an epoch of relatively low atmospheric CO_2 (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 C_4 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 CO_2 (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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