Ecdeiocoleaceae and Joinvilleaceae, sisters of Poaceae (Poales): evidence from *rbc*L and *mat*K data

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Abstract

Within Poales, the clade (Poaceae, Ecdeiocoleaceae, Joinvilleaceae) has previously received robust support from DNA sequence data from a range of genes. However, the question of which family (or set of families) is the sister group of Poaceae has not been answered with any degree of confidence, to date. Using *Ecdeiocolea monostachya* as the only representative of its family, other researchers have found the clade to resolve (although mostly with low levels of support) as (*Joinvillea* (*Ecdeiocolea*, Poaceae)). We now provide *mat*K and *rbc*L data for *Georgeantha hexandra*, the sole species in the other genus of Ecdeiocoleaceae, in addition to further sampling of other closely related Poales families. Analyses of our data support the alternative topology of (Ecdeiocoleaceae, Joinvilleaceae) as sister to Poaceae and also help to resolve the position of Flagellariaceae. *Flagellaria* groups with (Poaceae, Ecdeiocoleaceae, Joinvilleaceae) in a graminid clade that is sister to the restiid clade (Anarthriaceae, Restionaceae, Centrolepidaceae) in analyses of *mat*K or *mat*K + *rbc*L data. A high rate of base substitution has previously been noted in Poales; branch lengths in Ecdeiocoleaceae are comparable with those in Poaceae.

Introduction

The molecular phylogeny of Poales (APG 2003) has been the subject of much recent interest. Attention has particularly focussed on six plant families (Ecdeiocoleaceae, Joinvilleaceae, Flagellariaceae, Restionaceae, Centrolepidaceae and Anarthriaceae) that, with Poaceae, form the graminoid clade (Kellogg 2000, Bremer 2002) or 'core Poales' clade (Poales sensu stricto) of Barker et al. (2000) and Michelangeli et al. (2003). Of these, Ecdeiocoleaceae is endemic to southwestern Australia and was distinguished from Restionaceae by Cutler and Shaw (1965), largely on the basis of differences in culm anatomy (Cutler 1969). The species of Ecdeiocoleaceae, *Ecdeiocolea monostachya* F. Muell.[1] and *Georgeantha hexandra* B.G. Briggs & L.A.S. Johnson, both occur in semi-arid regions, whereas Joinvilleaceae and Flagellariaceae occur widely in the Old World tropics and Pacific.

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[1] Since this paper was submitted, a second species of *Ecdeiocolea* (still unnamed) has been distinguished (Briggs, unpublished). All data reported here for *Ecdeiocolea* refer to *E. monostachya*.

Various studies have identified Joinvilleaceae as a possible sister group of Poaceae (e.g. Campbell & Kellogg 1987, Chase et al. 1995, Stevenson & Loconte 1995, Kellogg 2000, GPWG 2001) while, until molecular data became available, Ecdeiocoleaceae were mostly regarded as more closely allied to Restionaceae than to Poaceae. Previously Briggs et al. (2000), on the basis of chloroplast DNA data, drew attention to Ecdeiocoleaceae as part of a small clade centred on Poaceae within the graminoid Poales. Subsequently Bremer (2002), using *rbc*L and *atp*B DNA sequence data, identified (Joinvilleaceae, Ecdeiocoleaceae, Poaceae) as a clade (here referred to as the JEP clade, following Hilu 2004). Bremer also concluded that Ecdeiocoleaceae is sister to Poaceae, a position which was also supported by Duvall (2003). Michaelangeli et al. (2003), using morphology with *atp*A and *rbc*L DNA sequence data, similarly placed Ecdeiocoleaceae as sister to Poaceae, noting that this grouping had low jackknife support but that alternative arrangements among the JEP families had even lower values. Hilu (2004) presented a hypothesis of chromosomal evolution in Poaceae, similarly placing Joinvilleaceae as sister to (Ecdeiocoleaceae, Poaceae). In Linder and Rudall's (2005) phylogenetic hypothesis of 'core Poales' the JEP clade (their graminid clade) is sister to a restiid clade. Thus the graminoid Poales (= 'core Poales') consist of the graminid (or JEP) clade and the restiid clade (Anarthriaceae sens. lat., Restionaceae, Centrolepidaceae), together with Flagellariaceae.

That Ecdeiocoleaceae is the single sister of Poaceae was further supported by results from a large *rbc*L data set for monocot taxa (Janssen & Bremer 2004) and a seven-gene data set including nuclear, plastid and mitochondrial sequences (Chase et al. 2006). Givnish et al. (2006), using *ndh*F data, however, placed *Ecdeiocolea* as sister to (*Joinvillea*, Poaceae) but noted that this topology collapsed to a trichotomy in the strict consensus tree. Preliminary maximum parsimony analysis of 17-gene plastid data provides unstable results concerning the sister group of the grasses, for different taxon samplings (JM Saarela & SW Graham, unpubl. data, pers. com.). In most of the studies mentioned, Ecdeiocoleaceae was represented only by *Ecdeiocolea*.

Flagellaria, the sole genus of its family, has been placed either as sister to the rest of the graminid clade (Bremer 2002, Chase 2004, Chase et al. 2006) or as sister to graminids plus restiids (Graham et al. 2006). The placement of *Flagellaria* within Poales has remained somewhat uncertain (Linder & Rudall 2005, Givnish et al. 2006).

Doyle et al. (1992) and Michelangeli et al. (2003) investigated three large inversions in the chloroplast DNA of Poales, and found that the 28 kilobase and 6 kb inversions are synapomorphies of Joinvilleaceae, Ecdeiocoleaceae and Poaceae, while the *trn*T inversion is apparently autapomorphic in Poaceae. These inversions therefore do not help to resolve the trichotomy of the JEP families, but for the record we report additional results from some other Poales taxa.

This study adds to the relevant DNA sequence data, especially by including the *mat*K sequence for *Georgeantha*. To investigate the difference between our conclusions and those of others who used different taxonomic sampling, we also present results from analyses that vary the taxonomic sampling and method of analysis of our data.

Materials and methods

Taxonomic and genomic sampling: this study analysed chloroplast DNA sequence data from *rbc*L and *mat*K of two species in Ecdeiocoleaceae, two in Joinvilleaceae and one of Flagellariaceae. Three samples of *Ecdeiocolea* and three of *Georgeantha* were sequenced, to check that the sequences used in the analysis were representative of each taxon; all have been submitted to GenBank but only one example from each taxon is included in the analyses[2]. Poaceae sampling reflected the clades indicated by the Grass Phylogeny Working Group (GPWG 2001) with *Anomochloa*, *Streptochaeta*, and *Pharus* representing basal clades, *Oryza*, *Melica* and *Triticum* the BEP clade (Bambusoideae, Ehrhartoideae, Pooideae), and *Aristida*, *Arundo*, *Danthonia*, *Zeugites*, *Zea* and *Eragrostis* from the PACCAD clade (Panicoideae, Arundinoideae, Centothecoideae, Chloridoideae, Aristoideae, Danthonioideae). Where sequences were available in GenBank, a representative was chosen from each subfamily recognised in the GPWG classification.

We have incorporated sequences from GenBank, as well as sequence data on *rbc*L that we (Briggs et al. 2000) and others have published previously, and we provide new sequence data for *rbc*L from a further nine taxa, and *mat*K from 15 taxa. Vouchers have been placed in the National Herbarium of New South Wales; particulars of the taxa (including authorities for names), vouchers and GenBank accession numbers are given in Table 1. For three genera of Poaceae (*Eragrostis*, *Melica* and *Aristida*) *rbc*L and *mat*K data were available in GenBank only from different species of the genus; the relevant species for each gene are listed in Table 1. We also experimented with the use of different sets of outgroup taxa.

DNA extraction and sequencing: DNA extraction, PCR conditions and primers used for *rbc*L have been described previously (Briggs et al. 2000). Amplification of *trn*K used two primers described by Johnson and Soltis (1995); the sequences of these are listed in Table 2, along with the internal sequencing primers used. The PCR program used was 95°C for 5 min, then 35 repetitions of (95°C for 30s, 55°C for 30s and 72°C for 2 min) with a final 10 min at 72°C, in a Corbett Research Palm Cycler. The polymerase enzyme used was 'BioTaq' (Bioline, Lukenwalde, Germany). Other details of PCR and subsequent sequencing are as given previously (Briggs et al. 2000). The portion of *rbc*L included consists of 1210 aligned bases, corresponding to positions 83 through 1292 of the gene in the *Oryza sativa* complete chloroplast DNA reference sequence (GenBank accession X15901).

Sequence alignment and analysis: sequences were aligned by CLUSTAL W, with modification by eye, using the program BioEdit (Hall 1999) version 5.0.6. Parsimony analyses used version 4.0b10 of PAUP* (Swofford 2001) for Power PC. The majority rule consensus trees represent the result of 1000 bootstrap replicates, from full heuristic searches with random taxon addition. The sets of sequence data for the regions of chloroplast DNA were analysed separately and in combination, using parsimony and

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[2]In *Ecdeiocolea monostachya* the three *rbc*L sequences were identical; for *mat*K two of the sequences were identical but differed from the third in having two separate single-codon insertions. In *Georgeantha hexandra* the *rbc*L and *mat*K sequences each showed a single-nucleotide substitution in one of the samples.

maximum likelihood (ML) criteria. ML analyses (with ML parameters estimated as part of the ML analysis) used either the default options or the TVM+I+G model selected by the Akaike information criterion (AIC) in Modeltest 3.7 (Posada & Crandall 1998). Since our findings for the sister group of Poaceae differ from those found in a number of other studies, and since Ecdeiocoleaceae was represented in those other studies by *Ecdeiocolea* alone, rather than by both genera of the family, we repeated some of the analyses omitting data either from *Ecdeiocolea* or from *Georgeantha*. In Fig. 1 the topology resulting from PAUP* analysis, from the consensus of 1000 bootstrap replicates, analysed using the criterion of maximum parsimony, is combined with relative branch lengths calculated using the DNAPARS program (in PHYLIP version 3.6) for mapping characters onto the topology, a procedure suggested by Felsenstein (2004). The representatives of Restionaceae, Anarthriaceae and Centrolepidaceae were used to root the tree. Percentage bootstrap support values for nodes are shown.

Inversions in the chloroplast genome: the approach of Doyle et al. (1992) and Michelangeli et al. (2003) was followed to determine the presence or absence of the 28 kb inversion previously reported in some Poales. This used primer pairs that span the ends of the inverted region and that are diagnostic for either the inverted or the uninverted sequence. The presence of the inversion was indicated by amplification with primer combinations *rps*14 +*trn*R at the *rps*14 end of the inversion or by G-UCC3' + G-GCC at the *trn*G-UCC end. Absence of the inversion was indicated by amplification with primers *rps*14 + G-GCC and G-UCC3' + *trn*R at the respective positions. Vouchers for the taxa studied are indicated in Table 1.

Results

Figure 1 illustrates our best inference of relationships of the taxa, based on the combined *rbc*L and *mat*K sequence data summarised in Table 3. Since *mat*K provided more parsimony informative characters than *rbc*L, the combined-data tree largely reflects the findings from *mat*K alone. The analyses gave the following results:

(1) The JEP clade is robustly supported with 100% bootstrap support in all parsimony analyses.

(2) All parsimony analyses that included both genera of Ecdeiocoleaceae gave bootstrap values of 95% or higher to the clade (*Ecdeiocolea*, *Georgeantha*).

(3) The topology ((Joinvilleaceae, Ecdeiocoleaceae) Poaceae) is supported when all relevant data from this study are included. When both *Ecdeiocolea* (Ec) and *Georgeantha* (Ge) are included in the analysis, the (J, (Ec,Ge)) clade receives 97% or 98% support (depending on the outgroups used) from *rbc*L + *mat*K data, or 93% support from *mat*K alone. When Ecdeiocoleaceae is represented by *Ecdeiocolea* alone, the (J,Ec) clade receives low or very low support: 70% from *rbc*L + *mat*K data, or 51% or 55% from *mat*K alone.
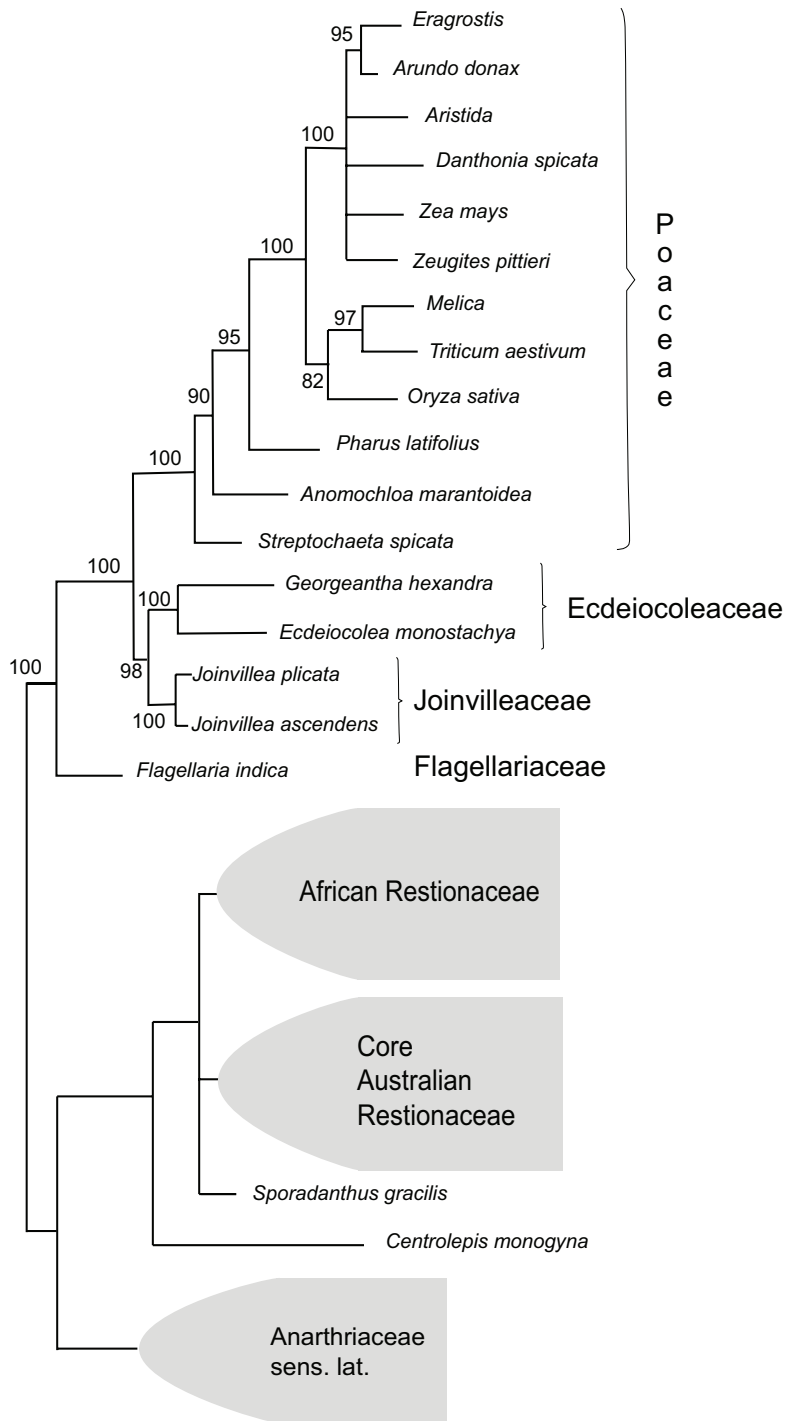
Fig. 1. Consensus tree from analysis of combined *rbc*L and *mat*K sequence data (see text). Percentage bootstrap support values for nodes are shown.

Table 1. Voucher information and GenBank accession numbers for taxa used in this study.

Source and voucher details are given for new sequences; vouchers are placed in the NSW herbarium, except for two collections by Linder et al. deposited at the Institute of Systematic Botany, University of Zurich (Z). Vouchers for study of the 28-kb chloroplast DNA inversion are marked*.

Taxon	*rbc*L	*mat*K	Source	Voucher
Anarthriaceae				
Anarthria prolifera R.Br.	DQ307438	DQ257499	*Meney s.n.*, Jarrahdale, W.A.	NSW415191
Hopkinsia adscendens B.G.Briggs & L.A.S.Johnson	AF148777	DQ257518	*Briggs 9342*, Oldfield River, W.A.	NSW391372*
H. anoectocolea (F.Muell.) D.F.Cutler	–	DQ257519	*Meney 920*, Arrowsmith R., W.A.	NSW364831*
Lyginia barbata R.Br.	AF148787	DQ257523	*Briggs 9321*, N of Cataby, W.A.	NSW391339
Centrolepidaceae				
Centrolepis monogyna (Hook.f.) Benth.	DQ307439	DQ257505	*Briggs 9514*, Lake Dobson, Tas.	NSW494429
Cyperaceae				
Scirpodendron ghaeri Merrill	AB0888323	AB088804		
Ecdeiocoleaceae				
Ecdeiocolea monostachya F.Muell	AF148773	DQ257528	*Meney T20*, S of Eneabba, W.A.	NSW364828*
	DQ307440	DQ257529	*Briggs 9638*, S of Cunderdin, W.A.	NSW716273
	DQ307441	DQ257530	*Perkins*, Watheroo National Park., W.A.	NSW709043
Georgeantha hexandra BG.Briggs & L.A.S.Johnson	DQ307442	DQ257531	*Briggs 9480*, Skipper Road, S of Arrowsmith R., W.A.	NSW 437363
	DQ307443	DQ257532	*Briggs 9653*, NE of Arrowsmith R., W.A.	NSW703380
	DQ307444	DQ257533	*Perkins*, Correy Road, N of Eneabba, W.A.	NSW709054
Flagellariaceae				
Flagellaria indica L.	DQ307445	DQ257515	*Marchant*, cult. Royal Botanic Gardens Sydney	NSW615132*
Joinvilleaceae				
Joinvillea ascendens Brongn. & Gris. subsp. *glabra* Newell	DQ307446	DQ257534	*Briggs 9672*, cult. ex New Caledonia	NSW612727
J. plicata (Hook.f.) T.K.Newell	DQ307447	DQ257535	*Briggs 9673*, cult. ex New Caledonia	NSW612730

Taxon	*rbc*L	*mat*K	Source	Voucher
Poaceae				
Anomochloa marantoidea Brongn.	AF021875	AF164381		
Aristida latifolia Domin	—	AF164413		
Aristida congesta Roem. & Schult. subsp. *barbicollis* (Trin. & Rupr.) de Winter	AJ746283	—		
Arundo donax L.	AJ746284	AF164408		
Danthonia spicata Roem. & Schult.	U31102	AF164409		
Eragrostis capensis Trin.	U31104	—		
E. grandis Hillebr.	—	AF 312342		
Melica altissima L.	—	AF164399		
M. uniflora Retz.	AJ746294			
Oryza sativa L.	X15901	X15901		
Pharus latifolius L.	AY357724	AF164388		
Streptochaeta spicata Schrad. ex Nees	AJ419949	AF164383		
Triticum aestivum L.	NC002762	NC002762		
Zea mays L.	X86563	X86563		
Zeugites pittieri Hack.	AY632374	AF144576		
Restionaceae				
Anthochortus graminifolius (Kunth) H.P.Linder	Hardy pers. comm.	Hardy pers. comm.	Linder et al. 7552	
Baloskion tetraphyllum (Labill.) B.G.Briggs & L.A.S.Johnson subsp. *tetraphyllum*	AF148761	DQ257501	Briggs 9120, Bicheno, Tas.	NSW365050
Calopsis fruticosa (Mast.)	Hardy pers. comm.	Hardy. pers. comm.	Linder et al. 7309	
Chordifex crispatus (R.Br) B.G.Briggs & L.A.S.Johnson	DQ307448	DQ257510	Briggs 9446, E of Wellstead, W.A.	NSW422605
Elegia cuspidata Mast.	AF148774	DQ257512	Romanowski s.n., cultivated	NSW255150
Empodisma minus (Hook.f.) L.A.S.Johnson & D.F.Cutler	DQ307449	—	Briggs 9508, Mt Wellington, Tas.	NSW494674
	—	DQ257513	Briggs 9135, Mt Tim Shea, Tas.	NSW264841
Eurychorda complanata (R.Br.) B.G.Briggs & L.A.S.Johnson	AF148790	DQ257514	Briggs 9136, Mt Tim Shea, Tas.	NSW264949
Lepyrodia glauca (Nees) F.Muell.	AF148785	DQ257521	Pate s.n., Brunswick Junction, W.A.	NSW423726
Platycaulos compressus (Rottb.) H.P.Linder	AY881464	AY881537		
Sporadanthus gracilis (R.Br.) B.G.Briggs & L.A.S.Johnson	DQ307450	DQ257525	Briggs 9263, W of Engadine, N.S.W.	NSW270154
Typhaceae				
Typha latifolia L.	L05464	AB088801		

Table 2. Sequencing Primers for amplication of *trn*K.

Name	Note	Sequence (5' – 3')
*trn*K–3914F (monocot)	a	TGGGTTGCTAACTCAATGG
MBK–F2		TATGAATGGAAAAAACAGCATGTCG
*trn*K–710F	b	GTATCGCACTATGTATCATTTGA
MBK–F3		GTATGGGGGCACCCTATTAGTAAACC
MBK–R2		TCGGCCCAGGTTGGTTTACTAATAGG
MBK–R3		GAAGTTAATCGTAAACAAGAAGATTG
MBKgeoR	c	TCACATTCCGACACATAGGAATTATATAGG
*trn*K–2R	b	AACTAGTCGGATGGAGTAG

(a) from Johnson and Soltis (1995), shortened at 5' end.
(b) from Johnson and Soltis (1995).
(c) designed particularly for *Georgeantha*.

When Ecdeiocoleaceae is represented by only *Georgeantha*, the (J,Ge) clade receives 74% or 79% from *rbc*L + *mat*K data. Using *rbc*L alone, the JEP clade is not resolved or resolves to discordant topologies with very low support.

(4) *Flagellaria* grouped with the JEP families, in a graminid clade that is sister to the restiid clade (Anarthriaceae, Restionaceae, Centrolepidaceae), in analyses of *mat*K or *mat*K + *rbc*L data. The clade (*Flagellaria* (JEP)) was found in ML analyses and received 87% or more support in parsimony analyses. Data from *rbc*L alone was insufficient to resolve the position of Flagellariaceae, which formed a trichotomy with the JEP and restiid clades in such analyses.

(5) When the results of parsimony and ML analyses were compared (Table 3), the interrelationships of the JEP families and of Flagellariaceae were identical under each analysis method, and did not depend on whether ML analyses used the default PAUP* settings (HKY) or the preferred model (TVM+I+G) found by MODELTEST. Including taxa from a range of outgroup families of Poales (Restionaceae, Centrolepidaceae, Anarthriaceae), rather than *Flagellaria* alone, increased support levels but varying the outgroups did not alter the resultant topologies for the critical nodes. Adding further outgroups from Cyperaceae and Typhaceae seemed to introduce more 'noise' as this reduced the support levels but did not alter the resultant topologies.

(6) We found no indels in *mat*K that assisted in resolving the JEP clade. There was a one-codon insertion shared by all members of that clade but absent in the other families. Our data (Table 4) conformed to the findings of Hilu and Alice (1999) who drew attention to variation in the 3′ end of *mat*K in Poaceae. There is a single base deletion in the majority of Poaceae, but *Anomochloa* retains a plesiomorphic condition, as do Joinvilleaceae and Restionaceae. Such a frame-shift indel is also absent from the other families studied, including Ecdeiocoleaceae. A nearby two-codon insertion occurring in the PACC grasses was absent in the other families. Restionaceae, Centrolepidaceae and part of Anarthriaceae (*Anarthria* but not *Hopkinsia* and *Lyginia*) show a one-codon deletion in this area. The codon (CAA) present at this position in *Hopkinsia* and *Lyginia* is not matched in other sequences and, in those two genera, a one-codon insertion may have followed a deletion that was shared by the three families.

Table 3. Results of analyses of *rbcL* and *matK* data separately and in combination, with variation in outgroups, method of analysis and in representation of Ecdeiocoleaceae.

Outgroup families are represented as follows: Flagellariaceae: *Flagellaria indica*; Anarthriaceae: *Anarthria prolifera, Hopkinsia adscendens, Lyginia barbata*; Restionaceae: *Anthochortus graminifolius, Baloskion tetraphyllum, Calopsis fruticosa, Chordifex crispatus, Elegia cuspidata, Empodisma minus, Eurychorda complanata, Platycaulos compressus, Sporadanthus gracilis*; Cyperaceae: *Scirpodendron ghaeri*; Typhaceae: *Typha latifolia*. Taxon abbreviations: Ec = *Ecdeiocolea*; Ge = *Georgeantha*; J = *Joinvillea*, Joinvilleaceae; P = Poaceae; other abbreviations: P = maximum parsimony; ML = maximum likelihood analysis using PAUP default values; ML * = Maximum Likelihood analysis using TVM+I+G model, selected by the Akaike information criterion in MODELTEST 3.7. Clade structure for the JEP clade, bootstrap support (BS) for parsimony analyses, consistency index (CI) and retention index (RI) are given.

Gene	Representation of Ecdeiocoleaceae	Total characters	Outgroup	Analysis	Variable characters — Informative	Variable characters — Uninformative	Clades	BS support for relevant node of (J,Ec,Ge,P) clade	CI	RI	BS support for (Ec,Ge) node
rbcL	Ec, Ge	1210	Flagellariaceae	P	143	86	P(J(Ec,Ge))	(J(Ec,Ge)) 60%	0.602	0.616	95%
	Ec			P	133	90	P(Ec,J)	(Ec,J) 58%	0.613	0.596	-
	Ge			P	136	88	Ge(J,P)	(J,P) 76%	0.615	0.598	-
matK	Ec, Ge	1672	Flagellariaceae	P	274	311	P(J(Ec,Ge))	79%	0.770	0.739	96%
	Ec			P	255	299	Ec(J,P)	62%	0.781	0.736	-
	Ge			P	253	300	P(J,Ge)	51%	0.781	0.730	-
rbcL + *matK*	Ec, Ge	2882		P	429	395	P(J(Ec,Ge))	(J(Ec,Ge)) 95%	0.711	0.700	100%
	Ec, Ge			ML	763	367	P(J(Ec,Ge))	(J(Ec,Ge)) 98%	0.607	0.793	100%
	Ec			P	747	360	P(J,Ec)	(J,Ec) 74%	0.615	0.801	-
	Ge			P	749	366	P(J,Ge)	(J,Ge) 73%	0.617	0.801	-
rbcL	Ec, Ge	1210	Flagellariaceae, Anarthriaceae, Restionaceae, Centrolepidaceae	P	218	76	P, J(Ec,Ge) / P(J(Ec,Ge))	(J(EG)P) 100%	0.529	0.777	96%
	Ec, Ge			ML	212	77	J,Ec,P	(J,Ec,P) 100%	0.533	0.778	-
	Ge			P	214	76	Ge(J,P)	(J,P) 70%	0.535	0.778	-
matK	Ec, Ge	1672		P	545	291	P(J(Ec,Ge)) / P(J(Ec,Ge))	(J(Ec,Ge)) 93%	0.640	0.812	100%
	Ec, Ge			ML	535	283	P(J,Ec)	(J,Ec) 55%	0.648	0.812	-
	Ge			P	535	290	P(Ge,J)	(Ge,J) 74%	0.651	0.813	-
rbcL + *matK*	Ec, Ge	2882		P	187	407	P(J(Ec,Ge)) / P(J(Ec,Ge))	(J(Ec,Ge)) 97%	0.583	0.767	97%
	Ec, Ge			ML *	801	407	P(J,Ec)	(J,Ec) 70%	0.590	0.773	-
	Ge			P	805	404	P(J,Ge)	(J,Ge) 79%	0.591	0.772	-
rbcL	Ec, Ge	1210	Flagellariaceae, Anarthriaceae, Restionaceae, Centrolepidaceae, Cyperaceae	P	231	103	P, J(Ec,Ge)	P, J,(Ec,Ge) 100%	0.526	0.759	97%
	Ec			P	226	104	J,Ec,P	(J,Ec,P) 100%	0.533	0.762	-
	Ge			P	228	102	Ge(J,P)	(J,P) 60%	0.531	0.758	-
matK	Ec, Ge	1672	Typhaceae	P	586	304	P(J(Ec,Ge))	(J(Ec,Ge))	0.606	0.771	91%
	Ec			P	575	299	P(J,Ec)	(J,Ec)	0.614	0.780	-
	Ge			P	578	302	P(J,Ge)	(J,Ge)	0.615	0.779	-

Table 4. Sequence for the 3´ end of *mat*K.

The stop codon is underlined (alignment largely following Hilu and Alice 1999).

Anarthriaceae
          Hopkinsia adscendens   CTGGTGAATCGTCAA———TAA
          Lyginia barbata   CTGGTGAATCCTCAA———TAA
          Anarthria prolifera   CTGGTGAATCAT————TAA
Centrolepidaceae
          Centrolepis monogyna   CTAGCAAATCTG————TAA
Cyperaceae
          Scirpodendron ghaeri   CTTATTAGTCATTTC————TAA
Ecdeiocoleaceae
          Ecdeiocolea monostachya   CTGGTGAATTATTCT———TAA
          Georgeantha hexandra   CTGGTGAATCATTCT———TAA
Flagellariaceae
          Flagellaria indica   CTGGTGAATCATTGG———TAA
Joinvilleaceae
          Joinvillea ascendens   CTGGTGAATCATTCT———TAA
          Joinvillea plicata   CTGGTGAATCATTCT———TAA
Poaceae
          Anomochloa marantoidea   CTGGTGAATTACTCT———TAA
          Aristida latifolia   CTGGTGAATC-CTCTTACTCTTAATTAA
          Arundo donax   CTGGTGAATC-CTCTTATTCTTAATTAA
          Danthonia spicata   CTGGTGAATC-CTCTTACTCTTAATTAA
          Eragrostis grandis   CTGGTGAATC-CCCTTACTCTTAATTAA
          Melica altissima   CTGGTGAACC-CTCT———TAATTAA
          Oryza sativa   CTGGTGAATC-CTCT———TAATTAA
          Pharus latifolius   CTGGTGAATC-CTGA———TAATTAA
          Streptochaeta spicata   CTGGTGAATCATTCT———TAATTAA
          Triticum aestivum   CTGGTGAATC-CTCT———TAATTAA
          Zea mays   CTGGTGAATC-CTCTTACTCTTAATTAA
          Zeugites pittieri   CTGGTAAATC-CTC TTACTTATAATTAA
Restionaceae (African)
          Anthochortus graminifolius   CTGGCGAATCTT————TAA
          Calopsis fruticosa   CTGGCGAACCTTTCATTT——TAA
          Elegia cuspidata   CTGGCGAATCCT————TAA
          Platycaulos compressus   CTGGCGAATCTTTCATTATGGATCATGA*
Restionaceae (Australian)
          Chordifex crispatus   CTGGCAAATCCG————TAA
          Baloskion tetraphyllum   CTAGCAAATCTG————TAA
          Empodisma minus   CTGGCAAATCTC————TAA
          Eurychorda complanata   CTGGCAAATCCG————TAA
          Sporadanthus gracilis   CTGGCAAATCTT————TAA
Typhaceae
          Typha latifolia   TTAGTGAACCATTGA———TAA

*there are 29 additional nucleotides in this sequence (not shown); terminating with a TGA stop codon.

Table 5. Presence of 28 kb inversion in the chloroplast genome: results from this study and previous reports on the same genera.

Family	Taxon	28 kb inversion	Previous reports
Anarthriaceae	*Anarthria prolifera*	absent	absent in *A. scabra* (Michelangeli et al. 2003)
	Hopkinsia adscendens	absent	
	Hopkinsia anoectocolea	present	
Ecdeiocoleaceae	*Ecdeiocolea monostachya*	present	present (Michelangeli et al. 2003)
	Georgeantha hexandra	present	
Flagellariaceae	*Flagellaria indica*	absent	absent (Doyle et al. 1992)
Restionaceae	*Lepyrodia glauca*	absent	

(7) The 28-kb inversion in the chloroplast genome was found to be present in three taxa (Table 5), while its absence in three other taxa was confirmed.

Discussion

Rate of nucleotide substitution: previous studies have drawn attention to the unusually high rate of nucleotide substitution, compared with other angiosperms, in the DNA of *rbc*L in Poaceae. Such long branch lengths in other Poales were noted by Bremer (2000) and are evident in the multi-gene analyses of Graham et al. (2006), Chase et al. (2006) and Givnish et al. (2006). Branch lengths in Ecdeiocoleaceae are similarly long, unlike those of Joinvilleaceae (Fig. 1).

Phylogenetic results: there is robust support in all trees for the major clades and relationships previously identified in Poales sens. strict. by Briggs et al. (2000), Bremer (2002), Michelangeli et al. (2003), Chase et al. (2006) and Graham et al. (2006). In particular, strongly supported clades here are (Poaceae, Ecdeiocoleaceae, Joinvilleaceae) and (Restionaceae, Centrolepidaceae). We also find robust support for the monophyly of Ecdeiocoleaceae. The two genera of Ecdeiocoleaceae, each regarded as monotypic until the very recent discovery of a second species of *Ecdeiocolea* (not yet named), show great similarity in vegetative and reproductive structures, including highly distinctive culm anatomy, shapes and textures of floral parts, seed ornamentation and an unusual reproductive biology (i.e. a form of monoecy with multiple switching between the production of female and male flowers along the length of the spike). Differences between *Georgeantha* and *Ecdeiocolea* include the former's trimerous flowers and dehiscent fruits. Michaelangeli et al. (2003) suggested that, if the indehiscent fruit type is homologous in *Ecdeiocolea* and Poaceae, these may be more closely allied than either of them is to *Georgeantha,* whereas Rudall et al. (2005) interpreted the dehiscence of *Georgeantha* fruits as the result of a character reversal. In view of the overall morphological similarity between *Ecdeiocolea* and *Georgeantha*, it is surprising that both *rbc*L and *mat*K show considerable divergence between them. Indeed, from *rbc*L data, an age estimate of more than 55 my BP for their divergence is given by Bremer (2002), and 73 my BP by Janssen and Bremer (2004), comparable to the age of crown Poaceae.

The trees from our *mat*K and our combined data agree with Bremer (2002), Chase (2004) and Chase et al. (2006) in finding Poales sens. strict. to divide into a graminid and a restiid clade, with *Flagellaria* associating with the JEP families to form the graminid clade, while (Anarthriaceae (Restionaceae, Centrolepidaceae)) form the restiid clade. By contrast, the insufficiently resolved *rbc*L tree placed *Flagellaria*, Anarthriaceae and (JEP families + Restionaceae including Centrolepidaceae) at a trichotomy, a position for *Flagellaria* more in agreement with the results from combined data of morphology, *atp*A and *rbc*L (Michelangeli et al. 2003). Graham et al. (2006) and Givnish (2006) found *Flagellaria* to be sister to a clade including both Poaceae and Restionaceae. Data from *rbc*L, *mat*K and *trn*L–F relevant to relationships within the restiid clade will be presented elsewhere.

Presence of 28-kb inversion in chloroplast genome: this inversion has previously been reported (Doyle et al. 1992, Michelangeli et al. 2003) as present in *Ecdeiocolea, Joinvillea* and all Poaceae sampled, but as absent in *Flagellaria* and *Anarthria*. Our findings are in agreement with these earlier reports for Ecdeiocoleaceae and Flagellariaceae.

There have been discordant findings in Restionaceae since these authors reported the inversion as present but Katayama and Ogihara (1996) reported it as absent in the restionaceous genera they studied; our evidence also indicated its absence in *Lepyrodia*. The results for the two species of *Hopkinsia* are discordant and this genus should be investigated further. The strong amplification in *H. anoectocolea*, indicating presence of the inversion, was unexpected since results for *H. adscendens* and *Anarthria* indicated that the inversion was not present.

Conclusion

Determining whether Ecdeiocoleaceae alone or (Ecdeiocoleaceae, Joinvilleaceae) is sister to Poaceae has a parallel in endeavours to determine whether the basal branch of the angiosperms is *Amborella* or (*Amborella*, Nymphaeales). Studies by Zanis et al. (2002) and Leebens-Mack et al. (2005) give a salutary warning against accepting as conclusive a topology with high bootstrap support but limited taxon sampling.

Our study supports the clade (Ecdeiocoleaceae, Joinvilleaceae) as sister to Poaceae. However, others (Chase 2004, Chase et al. 2006, Graham pers. com.) have investigated a wider range of genes in the Poales and found support for Ecdeiocoleaceae alone as the sister group. Adding *Georgeantha* to the sampling of Ecdeiocoleaceae, as in our study, greatly increases support for the ((Joinvilleaceae, Ecdeiocoleaceae) Poaceae) topology.

Acknowledgments

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