Phylogeny of *Elatostema* (Urticaceae) using chloroplast DNA data

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Abstract

Hadiah, Julisasi T.[1,2], Christopher J. Quinn[2] and Barry J. Conn[2] ([1]School of Biological, Earth and Environmental Sciences, University of New South Wales, Sydney, NSW 2052, Australia; [2] Royal Botanic Gardens Sydney, Mrs Macquaries Road, Sydney, NSW 2000, Australia) 2003. Phylogeny of Elatostema *(Urticaceae) using chloroplast DNA data.* Telopea *10(1) 235–246.* Phylogenetic analyses of the Urticales, based on chloroplast DNA data, support the monophyly of the Urticaceae, *Boehmeria*, *Pilea* and *Procris*, but not of *Elatostema*. Our result suggests that the circumscription of *Procris* is to be extended or included within *Elatostema*. At the tribal level, both Boehmerieae and Lecantheae appear paraphyletic, although this may be an artefact of the low taxon sampling. Preliminary analyses of relationships within *Elatostema* do not support the recognition of the subgenus *Pellionia*.

Introduction

Friis (1989 & 1993) provides a detailed comparison of morphological features of the Urticaceae at the familial, infrafamilial, and generic levels with a brief discussion of higher-level relationships based on previous classical taxonomic approaches. Recent phylogenetic studies involving the Urticaceae have concentrated on ordinal relationships. The circumscription of the Urticales has been relatively stable since the mid 1800s when Weddell (1856) included Artocarpeae, Cannabineae, Moreae, Ulmaceae and Urticaceae in the order. This classification was used by Thorne (1992) and Takhtajan (1997). Barbeyaceae was added by Dickison and Sweitzer (1970) and followed by Cronquist (1981), Dahlgren (1989), and Friis (1993). Cecropiaceae was proposed by Berg (1978) and placed close to Moraceae and Urticaceae. The reconstructed high-level phylogenies of Chase et al. (1993), using the DNA sequences of the chloroplast gene *rbc*L, support the monophyly of the Urticales with Cannabaceae, Moraceae, Ulmaceae and Urticaceae included. However, subsequent analyses including additional loci (Angiosperm Phylogeny Group 1998; Soltis et al. 2000) and non-molecular data (Judd et al. 1999) have shown this group to be nested within the Rosales. All these authors consider Cannabaceae, Celtidaceae, Cecropiaceae, Moraceae and Ulmaceae to be the closest families to Urticaceae.

Weddell (1854, 1856, 1869) revised the familial and infrafamilial classification of Urticaceae devised by Gaudichaud (1830) and recognised five tribes, namely, Lecantheae, Urereae (= Urticeae *sensu* Friis 1989), Boehmerieae, Parietarieae and Forskohleae (= Forsskaoleae *sensu* Friis 1989), renaming the tribe Lecantheae to Procrideae (Weddell 1856). Friis (1993) accepted Weddell's circumscription of these five tribes; however, he reversed the name Procrideae to Lecantheae (as accepted here). Friis (1989, 1993) characterised the Lecantheae as having staminodes that eject the mature achenes; leaves which are opposite, anisophyllous to completely reduced; intrapetiolar and fused stipules; and uniformly linear cystoliths. The Urticeae was characterised by the presence of stinging hairs. However, he questioned the distinctiveness of the other three tribes, and suggested further work may lead to a taxonomic rearrangement at the tribal level (Friis 1989).

Phylogenetic reconstruction of the Urticaceae using morphological data (Beaman 2000, Fig. 3-3), as part of a study of *Elatostema* from Mt Kinabalu (Malaysia), provided support for the monophyly of Lecantheae and Urticeae, but suggested that the Boehmerieae is polyphyletic. The Lecantheae consists of seven genera (Friis 1993), including *Elatostema*, the focus of our study. The genus consists of approximately 300 herbaceous to shrubby species (Friis 1993) that are characterised by having the female flowers arranged on a flattened discoid or lobed receptacle.

Schröter and Winkler (1935) recognized four subgenera within *Elatostema*, namely *Elatostema* (as '*Euelatostema*'), *Elatostematoides*, *Pellionia* and *Weddelia*, based on several features, but particularly the nature of leaves, stipules, inflorescence, and presence and form of the receptacle. Friis (1989, 1993) made no comment on the subgenera, but the analyses of Beaman (2000, 2001) did not support the arrangement.

This paper summarises the preliminary evaluation of different regions of the chloroplast genome for estimating relationships within Urticaceae, in general, and within *Elatostema*, in particular. As part of our continuing Urticaceae research program, we also aim to test the monophyly of the Lecantheae and *Elatostema*.

Materials

Plant materials used for the molecular work were either collected specifically for this project (namely from Indonesia — Sumatera, Java and Bali; and Australia — New South Wales, including Lord Howe Island) or are part of the horticultural collections at Royal Botanic Gardens Sydney. Fresh leaf material, particularly from the young shoots, cleaned and stored in airtight plastic bags with silica gel, was used for DNA analysis. These samples were stored at -20°C. The voucher specimens for DNA extracts are listed in Appendix 1 (all held at NSW).

Methods

Three regions of the chloroplast genome were selected for this study. The *rbc*L gene was chosen to test the monophyly of the family because of the availability in GenBank of sequences for representatives of several of the related families. Two potentially more informative regions were chosen to examine relationships within the family: the *atp*ß-*rbc*L intergenic spacer and a region including the *trn*L intron, the *trn*L-F intergenic spacer, and the intervening *trn*L exon. For simplicity, the latter is henceforth referred to as *trn*.

DNA was extracted from 0.2-0.25 g silica gel dried leaves and purified using the protocol of Gilmore et al. (1993). The three regions were amplified by polymerase chain reaction (PCR) in an FTS-4000 Thermal Sequencer (Corbett Research, Mortlake NSW) using 20 µM of the primers as listed in Appendix 2. All the PCR products were purified using CONCERT™ Rapid PCR Purification System (protocol provided by manufacturer). The cleaned PCR products were auto-sequenced at SUPAMAC (Sydney University Prince Alfred Macromolecular Analysis Centre). The DNA sequences were edited and aligned using Sequencher 3.1.1. (Gene Codes Corp., Inc., Ann Arbor, Michigan) with subsequent manual adjustment. Sequences were then viewed in MacClade Version 4.03 (Maddison & Maddison 2001) to assist with the positioning of segments affected by insertion/deletion mutations (indels). Deleted segments were treated as missing data in the analyses.

Our data for *rbc*L and *trn* were supplemented by sequences of the following taxa obtained from GenBank: *Celtis sinensis* (Ulmaceae), *Dorstenia psilurus*, *Ficus pretoriae, Morus alba* (2 accessions) and *M. rubra* (Moraceae), *Cannabis sativa* and *Humulus lupulus* (Cannabaceae) as indicated in Appendix 1. Since Chase et al. (1993, figs 11B & 16) and Soltis et al. (2000, fig. 7) concluded that the Urticaceae, Cannabaceae, Moraceae and Ulmacaeae (Celtidaceae *sensu* Soltis et al.) form a strongly supported clade, species from the latter three families have been used for outgroup comparison in the *rbc*L analysis. This analysis was used to test the monophyly of Urticaceae. Outgroup choice for each of the other two data sets was based on the *rbc*L analysis, as set out below.

Heuristic searches were performed in PAUP* Version 4.0b10 (Swofford 2002) using tree bisection reconnection branch-swapping and the MULPARS option, with all characters equally weighted to find the most parsimonious trees. Analyses involved 100 replicates of random taxon addition in order to search for multiple islands of equally parsimonious trees. Branch lengths for trees were calculated using the ACCTRAN (accelerated transformation optimisation) option in PAUP. Relative support for the clades identified by parsimony analysis was estimated by jackknife with 10000 replicates of fast stepwise addition using 33% character deletion and 'emulate jac resampling'.

Results

The *rbc*L database

Sequences were generated for 9 ingroup taxa from the Urticaceae, and another two ingroup and seven outgroup sequences were added from GenBank. A total of 1346 aligned positions was included in the analyses, of which 226 (16.8%) were variable and 140 (10.4%) parsimony informative. Missing data constituted 6.5% of the database, the taxon with the most missing data being *Ficus pretoriae* (33%). Initial heuristic analyses gave a topology that did not accord with the family level relationships and cast doubt on the identity of two of the outgroup sequences obtained from GenBank: *Morus alba* L01933 and *Dorstenia psilurus.* A BLAST search (NCBI November 2002) placed the former among a group of *Prunus* sequences (Rosaceae), and the latter among Rhamnaceae. A further outgroup sequence belonging to *Prunus persica* (Table 1) was obtained from GenBank and added to the data set, and the analysis repeated using the two Rosaceae sequences as root.

Heuristic search found a single island of two trees of 374 steps, consistency index (CI) = 0.606 without uninformative characters, retention index (RI) = 0.708, and rescaled consistency index (RC) = 0.496. The strict consensus tree is shown in Figure 1. The names of the misidentified taxa are shown within inverted commas. There is strong support (95% jackknife) for a sister relationship between Urticaceae and the clade comprising Moraceae, Cannabaceae and Ulmaceae. The two sequences of each of *Elatostema* and *Procris* are strongly grouped (jackknife support $\geq$ 95%), as are the four sequences of *Boehmeria* (94% support), and there is 82% support for the monophyly of Urticaceae. *Pilea pumila* is placed sister to *Urtica dioica* (89% support) rather than with the other genera of the tribe Lecantheae, *Elatostema* and *Procris*. *Myriocarpa longipes*, of the Boehmerieae, is placed closer to all four of the above genera (80% support) than to the *Boehmeria* clade.

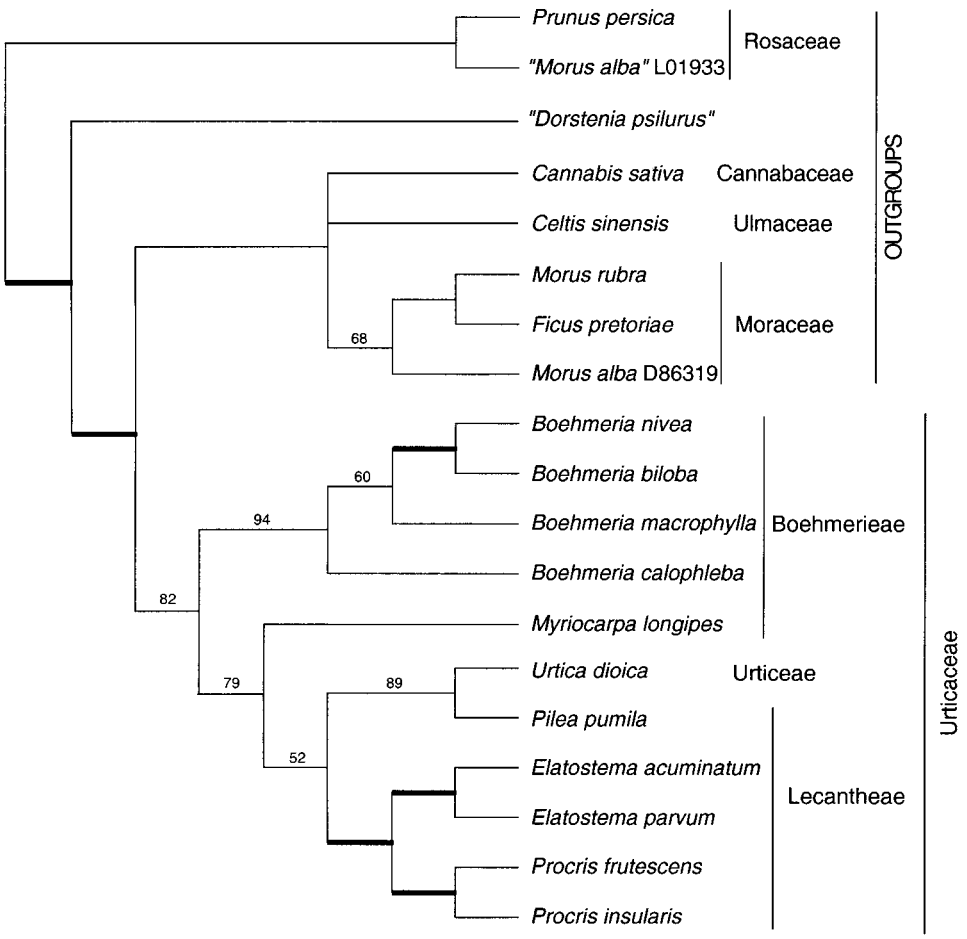

Fig. 1. Strict consensus of two equally parsimonious trees of 374 steps found from heuristic search of the *rbc*L data. CI = 0.606 excluding uninformative characters; RI = 0.708; RC = 0.496. Thick branches received >95% support; other jacknife values >50% shown above the clades.

The *atp*ß-*rbc*L database

Fourteen species of Urticaceae were sequenced, thirteen representing Lecantheae (*Elatostema*, *Procris* and *Pilea*), and *Myriocarpa longipes* from the Boehmerieae *sensu* Friis (1993). The latter was used to root the analysis. Alignment required numerous indels ranging from 1-69 bp. The aligned data constituted 962 positions. There were 168 variable positions (17.5%), of which 84 (8.7%) were potentially informative. Six potentially informative indels, ranging from 1-10 bp, were scored as additional characters (sequence present/absent) and added to the database. Missing data constituted 14.9% of the data set.

Heuristic search found a single island of ten trees of 191 steps, CI = 0.922 excluding uninformative characters, RI = 0.948, RC = 0.908. The strict consensus of these trees is shown in Figure 2. The distributions of informative indels have been mapped on the tree. Both *Elatostema* and *Procris* are very strongly supported as monophyletic groups (100% and three and two indels, respectively), and there is 95% support for a sister relationship between them.

The *trn* database

Sequences were generated for 24 taxa representing 16 species or species groups of *Elatostema* and eight other species of Urticaceae. Sequences of two outgroup species, namely *Humulus lupulus* and *Cannabis sativa*, were taken from GenBank. A total of 1108 aligned positions were included in the analyses, which included 582 base pairs (bp) of the *trn*L intron, 50 bp of the *trn*L exon, and 449 bp of the *trn*L-F intergenic spacer (the last 4 bp of the spacer were omitted). Alignment required numerous indels involving from 1-101 bp. Missing characters constituted 16.2% of data, with the taxon having the highest proportion of missing data being *Cannabis sativa* (39.6%). Thirty-one potentially informative indels, ranging from 1-51 bp, were scored as sequence present or absent and added to the database. There were 431 (38.9%) variable positions, 258 of which (23.3%) were potentially informative.

Heuristic search found a single island of two equally parsimonious trees of 663 steps, CI = 0.733 excluding uninformative characters, RI = 0.846, RC = 0.688. The strict consensus of these trees is shown in Figure 3. The distributions of informative indels have been mapped on this cladogram (Fig. 3). The species pairs representing *Boehmeria*, *Pilea* and *Procris* are each strongly grouped (100%), but *Elatostema* appears paraphyletic, with *E. curtisii* and *E. repens* placed sister to *Procris* with 99% support, whereas the remaining members of *Elatostema* constitute a very robust clade (100% support). There is good support (91%) for a sister relationship between these two clades. Once again, *Urtica dioica* is placed sister to *Pilea*, but jackknife support for this relationship is very weak (54%).

Discussion

Friis (1993) provides a detailed discussion of morphological characters of the Urticaceae. He circumscribed the family as having basal ovaries and stamens that are elastic and reflexed (Friis 1989, 1993). However, he does not provide a phylogenetic interpretation of these data. The monophyly of the Urticaceae was tentatively supported by Beaman (2000), based on morphological characters.

The analysis of the *rbc*L data, which places all Urticaceae within a clade that is sister to the five taxa belonging to three of the other five families of the order Urticales *sensu* Cronquist (1981), provides support for the concept of the family. The ingroup clade, which comprises 11 sequences drawn from six genera and three of the five tribes, receives 82% jackknife support. The current tribal arrangement, however, receives no

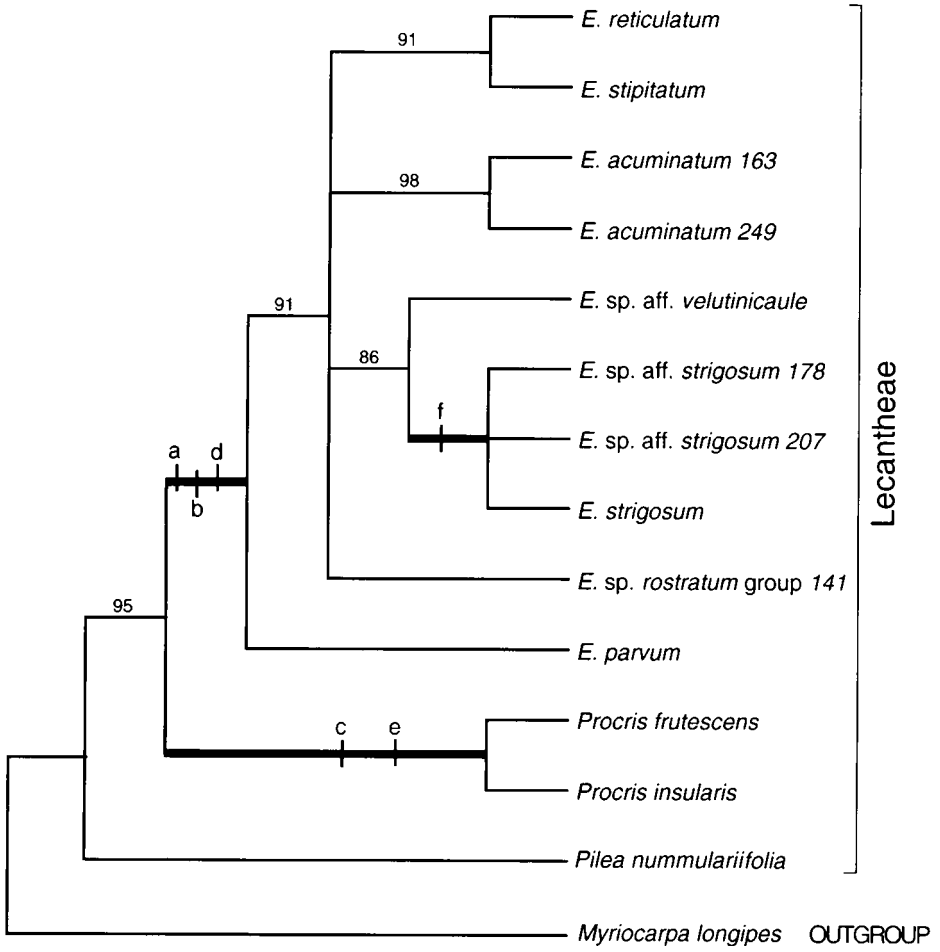

Fig. 2. Strict consensus of the 10 equally parsimonious trees of 191 steps found from heuristic search of the *atp*ß-*rbc*L spacer data set; CI = 0.922 excluding uninformative characters; RI = 0.948; RC = 0.908. Thick branches received 100% jackknife support; other values > 50% shown above the branches. Distributions of indels a-f are mapped on the tree. *E.*, *Elatostema*.

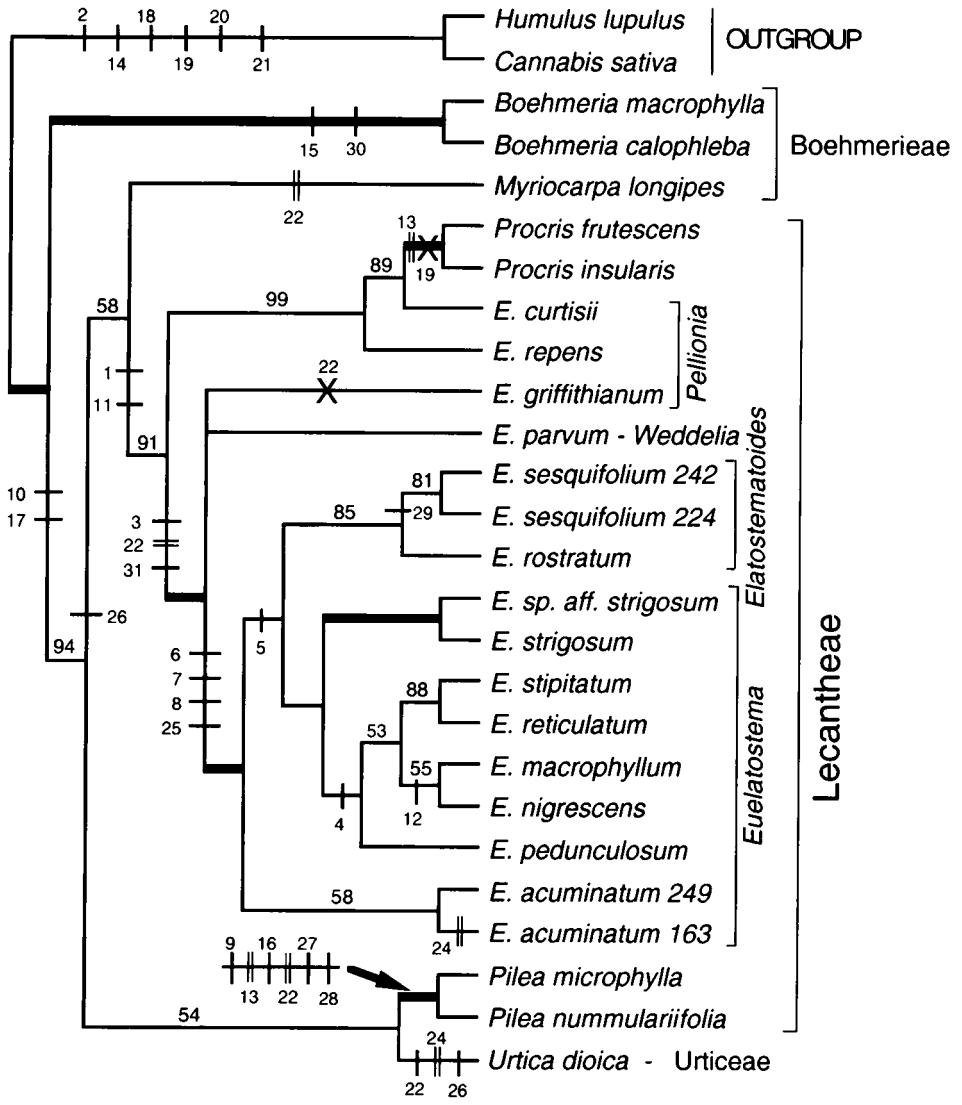

Fig. 3. Strict consensus of two equally parsimonious trees of 663 steps found from heuristic search of the *trn* data set; CI = 0.733 excluding uninformative characters; RI = 0.846; RC = 0.688. Thick branches received 100% jackknife support; other values >50% shown above the branches. Distributions of 31 scored indels have been mapped on the tree; single bar indicates unique origin; double bar indicates homoplasy; X indicates reversal. *E.*, *Elatostema*.

support. In both Figures 1 and 3, the Lecantheae (*Elatostema*, *Pilea* and *Procris*) and Boehmerieae (*Boehmeria* and *Myriocarpa*) are paraphyletic. Constraint analyses of the *trn* data set revealed that an extra 15 steps are required over and above the most parsimonious tree to render the Lecantheae monophyletic, and a total of 31 extra steps are needed to make both tribes monophyletic. It can be concluded, therefore, that there is considerable strength in these data to reject the present tribal arrangement of these genera. The grouping of *Pilea* with *Urtica*, however, which is apparent in both analyses, may well be an artefact of the low taxon sampling. It is only weakly supported on the *trn* data (54%), and both genera are on very long terminal branches (data not presented here).

The monophyly of *Boehmeria*, *Pilea* and *Procris* received high levels of jackknife support in all the analyses where more than one species was included, but the more extensive sampling of *Elatostema* in the *trn* analysis revealed it to be paraphyletic with respect to *Procris*. Support for the grouping of *Elatostema curtisii* and *E. repens* with *Procris* is very robust (99%).

Support for the '*Elatostema*-*Procris*' clade is high in all three data sets. The placement of the latter genus within *Elatostema* in the *trn* analysis, supports the broader concept of *Elatostema* adopted by Hallier (1896) and Winkler (1922). An alternative conclusion is that the current circumscription of *Procris* should be extended such that this group could be maintained as a separate genus. It is clear from Figure 3 that even as a subgenus, the limits of *Procris* need to be extended to include further species (eg. *Elatostema curtisii* and *E. repens*) currently assigned to *Elatostema*. The robust grouping (100%) of *E. griffithianum* with species of subgenera *Elatostema*, *Elatostematoides* and *Weddelia* indicates that the morphological basis for the recognition of subgenus *Pellionia* (Schröter & Winkler 1935 & 1936) (or as a distinct genus – as classified by Weddell 1856, Robinson 1910, Friis 1989), at least, is not supported by the molecular data. Beaman (2000, 2001), using morphological features, also concluded that subgenus *Pellionia* was not distinct from the other subgenera. Furthermore, the current circumscriptions of the first three subgenera are also not supported by molecular data.

Overwhelmingly, the distributions of indels are congruent with the estimate of the phylogeny obtained primarily from the substitution data, and they can be seen to support many of the clades: e.g. both species of *Pilea* are characterised by four unique indels (9, 16, 27 & 28) and another two (13 & 22) that also arise on other lineages (Fig. 3). All six of the informative indels in atpß-*rbc*L required only a single origin when their distributions were mapped on the strict consensus tree (Fig. 2), and only three of the 31 informative indels (13, 22 & 24) in the *trn* region required more than one origin (Fig. 3). Two of these (indels 13 and 24) involved the gain or loss of a single base pair from non-coding regions. Multiple origins of such indels in intergenic spacers have been frequently observed (e.g. Golenberg et al. 1993, Lowrey et al. 2001). Indels 19 and 22 required reversals (Fig. 3), but resolution of the trichotomy so that *Elatostema parvum* diverged after *E. griffithianum* would remove the need for the reversal in the latter case. The outgroup (Cannabaceae) differ from the ingroup by six indels (2, 14, 18, 19, 20 & 21), although indel 19 (a 6 bp insertion) has been subsequently lost in both species of *Procris*. This case of homoplasy (reversal or parallel origins) is interesting, since the indel is not a duplication of adjacent sequence, a type that has been observed to be common in spacer regions (Golenberg et al. 1993, Kelchner & Clark 1997). It is possible that secondary structure of the *trn*L-F spacer region may be responsible for the loss of the inserted region in its entirety, although there was no evidence of complementary segments of sequence on either side of the insertion which might promote the formation of a loop (Kelchner & Wendel 1996).

Sequences for all three regions could be confidently aligned across the family. Both the *trn* and *rbc*L data sets could be rooted outside the family, and yielded good resolution of generic relationships. The latter, however, provided only low levels of variability: e.g. the uncorrected pairwise sequence divergence between *Elatostema acuminatum* and *E. parvum* was only 0.7%. As a result, jackknife support for clades was often relatively low even in this small taxon set. It is concluded that this region of the chloroplast genome is insufficiently variable to provide robust resolution of relationships within the genus. The *trn* region provided the highest proportion of variable characters: the uncorrected pairwise divergence between *E. acuminatum* and *E. parvum* was 5.1%. Hence, this is the most promising of the three regions trialled here for resolving interspecific relationships within *Elatostema*. Even within this region, however, relationships were not fully resolved in a very limited taxon sample (Fig. 3), and pairwise divergences between species are frequently very low: e.g. 0.02% for *E. reticulatum* cf. *E. stipitatum*, 0.04% for *E. rostratum* cf. *E. sesquifolium*. It therefore appears that robust resolution of species relationships within the genus will require a more variable region of DNA. The nuclear encoded intergenic transcribed spacer region of the rDNA is currently being investigated for this purpose.

Finally, the recognition of the misidentification of two of the outgroup sequences highlights the caution that must be exercised about the authenticity of sequences lodged in GenBank and the importance of including voucher details when sequences are lodged — in this case no voucher was provided by either author.

Conclusion

Phylogenetic analyses of the Urticales, based on chloroplast DNA sequences, provided support for the monophyly of the Urticaceae, but not for the tribe Lecantheae. However, the apparent paraphyletic nature of the tribe may be an artefact of low taxon sampling, particularly in the Urticeae. Although *Boehmeria* is monophyletic, the Boehmerieae is polyphyletic, with the tribal position of *Myriocarpa* uncertain. The genus *Elatostema*, a member of the Lecantheae, has been shown to be paraphyletic, having the segregate genus *Procris* embedded within it. The preliminary analyses of infrageneric relationships within *Elatostema* do not support the recognition of the subgenus *Pellionia*.

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Appendix 1. List of voucher specimens for DNA extracts and GenBank numbers for sequences. (Classification of Urticaceae follows Friis 1989, 1993).

Taxa	Voucher No.	*rbc*L	*atp*B-*rbc*L	*trn* region
CANNABACEAE				
Cannabis sativa		AJ390068		AJ390367
Humulus lupulus				AB033889 — intron AB033890 — spacer
MORACEAE				
"Dorstenia psilurus"		AJ390066		
Ficus pretoriae		AJ390067		
Morus alba		D86319		
"M. alba"		L01933		
M. rubra		U06812		
ROSACEAE				
Prunus persica		AF206813		
ULMACEAE				
Celtis sinensis		D86309		
URTICAEAE				
Boehmerieae				
Boehmeria biloba		AJ390069		
B. calophleba	Hadiah 393	AY208700		AY208723
B. macrophylla	Hadiah 394	AY208701		AY208722
B. nivea		AJ235801		
Myriocarpa longipes	Hadiah 395	AY208705	AY208720	AY208724

Appendix 1. cont.

Taxa	Voucher No.	*rbc*L	*atp*B-*rbc*L	*trn* region
Lecantheae				
Elatostema–Elatostemoides				
E. rostratum	Hadiah 144			AY208743
E. rostratum group	Hadiah 141		AY208714	
E. sesquifolium	Hadiah 224			AY208742
E. sesquifolium	Hadiah 242			AY208741
Elatostema – Elatostema				
E. acuminatum	Hadiah 163		AY208710	AY208745
E. acuminatum	Hadiah 249	AY208702	AY208711	AY208744
E. macrophyllum	Hadiah 245			AY208739
E. nigrescens	Hadiah 256			AY208740
E. pedunculosum	Hadiah 312			AY208738
E. reticulatum	Perkins 00/01		AY208708	AY208737
E. stipitatum	Perkins 00/02		AY208709	AY208736
E. strigosum	Hadiah 159		AY208717	AY208735
E. sp. aff. *strigosum*	Hadiah 178		AY208715	AY208734
E. sp. aff. *strigosum*	Hadiah 207		AY208716	
E. sp. aff. *velutinicaule*	Hadiah 183		AY208713	
Elatostema – Pellionia				
E. curtisii	Hadiah 427			AY208731
E. griffithianum	Hadiah 351			AY208732
E. repens	Hadiah 445			AY208730
Elatostema – Weddelia				
E. parvum	Hadiah 154	AY208703	AY208712	AY208733
Pilea microphylla	Hadiah 398			AY208726
P. nummulariifolia	Hadiah 389		AY208721	AY208727
P. pumila		AF206811		
Procris frutescens	Hadiah 149	AY208704	AY208718	AY208728
P. insularis	Hadiah 390	AY208706	AY208719	AY208729
Urticeae				
Urtica dioica	Hadiah 391	AY208707		AY208725

Appendix 2. Primers use for PCR (P) and sequencing (S); F, forward; R, reverse.

Region	Use	Primer	Reference or sequence
	P/F	*rbc*L 1	GGGATTTATGTCACCACAAACAGA – P. Gadek, unpubl.
	P/R	*rbc*L 2	GATCTCCTTCCATACTTCACAAGC – P. Gadek, unpubl.
*rbc*L	S/F	861	TGGACCACTGTTTGGACCGA – P. Gadek, unpubl.
	S/F	381	GCAGTTATTGACAGACAAAGAAATCATGGT – P. Gadek, unpubl.
	S/R	497	ACCATGATTCTTCTGCCTATCAATAACTGC – P. Gadek, unpubl.
	P/F	377	Crayn & Quinn (2000)
	P/R	520	O'Brien et al. (2000)
*atp*ß-*rbc*L	S/F	2603	Crayn & Quinn (2000)
	S/R	2604	Crayn & Quinn (2000)
	S/R	2607	Crayn & Quinn (2000)
	PS/F	B49317	Taberlet et al. (1991)
	PS/R	CalTabF	GTCCTCTGCTCTACCAACTG – A. Perkins, unpubl.
*trn*L-F	PS/R	A50272	Taberlet et al. (1991)
	S/F	AdTabB2	Briggs et al. (2000)
	S/R	A49855	Taberlet et al. (1991)