Phylogenetic relationships in the genus *Lichtensteinia* (Apiaceae) based on morphological, anatomical and DNA sequence data

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Abstract

Evolutionary relationships within the poorly known and anomalous South African endemic genus *Lichtensteinia* were elucidated. Phylogenetic analysis of morphological and anatomical characters suggests that there are two main groups of species, viz. the *L. obscura–L. globosa–L. interrupta* (including *L. kolbeana*) clade and the *L. latifolia–L. trifida–L. lacera–L. crassijuga* clade. Furthermore, *Lichtensteinia* is not monophyletic, with the former group allied weakly with the Namibian endemic genus *Marlothiella* and the latter group allied strongly with *Annesorhiza macrocarpa*. In contrast, the results of phylogenetic analyses of nrDNA ITS and cpDNA trnQ-5'trnK sequences, separately and combined, as well as the results of a total evidence analysis of all available data, suggest three main groups of species in a monophyletic *Lichtensteinia*: the aforementioned *L. latifolia–L. trifida–L. lacera–L. crassijuga* complex, a clade comprising *L. interrupta* and *L. globosa*, and *L. obscura*. DNA sequence data, however, are not currently available for *Marlothiella*. The new species *L. globosa* B.-E. Van Wyk and P.M. Tilney is sister group to *L. interrupta* in the molecular analyses; in the analysis of morphological/anatomical data, however, the relationships among *L. globosa*, *L. interrupta* and *L. obscura* are equivocal. *Lichtensteinia lacera* and *L. trifida* are each not monophyletic based on the molecular phylogenies. The various populations of *L. lacera* and *L. trifida* examined are morphologically quite distinct, with large differences observed among populations but limited variation within populations. The size and shape of the leaves and of the marginal teeth, as well as the presence and length of setae, were found to be of diagnostic value in distinguishing among the species.
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1. Introduction

Lichtensteinia Cham. and Schltdl. is an anomalous group of seven perennial herbs endemic to South Africa, occurring mainly in the Cape region. These plants are of ethnobotanical interest. Several reports on the use of the roots of *L. interrupta* (Thunb.) Sond. in traditional medicine (mainly for respiratory ailments) are recorded by Watt and Breyer-Brandwijk (1962). The roots of this species and those of *L. beiliana* Eckl. and Zeyh. [now known as *L. obscura* (Spreng.) Koso-Poljansky] are reported to be used for making a narcotic drink.

Lichtensteinia has remained relatively unstudied since the taxonomic treatment by Sonder (1862). A synopsis by Burtt (1991) clarified some nomenclatural questions but did not include any detailed morphological or anatomical information. The fact that *Lichtensteinia* has remained poorly known can be attributed to several factors. Most species are adapted to summer drought conditions by having essentially proteranthous leaves. This means that specimens with mature fruits are frequently leafless or have shriveled leaves which make their identification difficult. To collect complete reproductive and vegetative material from the same population, more than one visit is usually necessary. Not only are some of the species poorly represented in herbarium collections, but leaves and flowers or fruits from different species are sometimes mounted together on the same herbarium sheet. Furthermore, many specimens in herbaria are misidentified, partly because most of the species are exceptionally variable in leaf shape. In addition, the occurrence of distinct regional forms creates much uncertainty about the circumscription and appropriate rank of taxa within the genus (Burtt, 1991).

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The genus was hitherto classified in Apiaceae subfamily Apioideae and was placed in the tribe Ammineae (Apieae) subtribe Carinae by Drude (1897–98) and tentatively in the tribe Apieae by Pimenov and Leonov (1993). However, the position of *Lichtensteinia* within the Apiaceae is not clear since it shares several characters with both subfamilies Saniculoideae and Apioideae. Its traditional placement among the apioid umbellifers was based on a superficial acceptance of the apioid inflorescence without considering the numerous characters which it shares with genera of the Saniculoideae. These include proteranthous leaves, toothed leaf margins (some with marginal setae), enormous rib oil ducts in the fruits, an absence of vittae, and rounded cotyledons. Burtt (1991) suggested that the position of the genus within the family "certainly merits re-examination". Liu et al. (2003) suggested the transfer of *Lichtensteinia* into the Saniculoideae, but this proposal was rejected by Calviño and Downie (2007) on the basis of molecular evidence, which places *Lichtensteinia* as a sister group to the rest of the Apioideae. Moreover, many of the morphological characters once thought to unite *Lichtensteinia* with Saniculoideae have been interpreted as plesiomorphic or ambiguously reconstructed as inferred by phylogenetic analysis of molecular data (Calviño et al., 2006; Calviño et al., 2008).

In this paper, we elucidate phylogenetic relationships within the poorly known genus *Lichtensteinia* using morphological, anatomical, and molecular evidence. We survey morphological and anatomical characters previously considered important in delimiting species and analyze these data phylogenetically to reveal synapomorphies. The results of phylogenetic analyses of DNA sequences from both chloroplast and nuclear genomes are also presented, as are the results of analyses of combined molecular and morphological/anatomical data. These results are important, not only as a basis for a taxonomic revision of the genus, but also within the context of new circumscriptions at the subfamily level.

Table 1
Morphological and anatomical characters and character states used in the cladistic analysis

Characters	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
Taxa																									
Dracosciadium																									
Italae	1	0	?	?	?	0	1	0	0	0	?	1	0	1	0	0	0	0	1	1	1	0	0	0	0
Annesorhiza macrocarpa	1	1	0	0	0	1	0	0	0	0	?	1	0	1	0	0	1	1	0	1	?	0	0	1	0
Marlothiella																									
*gummifera**	0	?	?	?	?	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0
L. obscura	1	1	1	0	0	1	0	0	0	0	0	1	1	0	0	0	0	1	0	1	1	1	1	0	0
*L. interrupta***	1	1	1	0	0	1	0	0	0	0	0	1	1	1	0	1	0	0	1	0	1	1	1	0	0
L. globosa	1	1	1	0	0	1	0	0	0	1	0	1	0	0	0	1	0	0	1	0	0	1	1	0	0
L. crassijuga	1	0	1	2	0	1	0	0	1	0	1	0	1	1	1	0	1	1	0	1	0	1	0	1	1
L. latifolia	1	0	0	1	0	1	1	1	1	0	1	0	1	1	1	0	1	1	0	1	0	1	0	1	1
*L. trifida****	1	0	0	2	1	1	1	1	1	1	1	0	1	1	1	0	1	1	0	1	0	1	0	1	1
L. lacera	1	0	0	2	1	1	1	1	1	1	1	0	1	1	1	0	1	1	0	1	0	1	0	1	1

Characters and character states described and illustrated in Liu et al. (2007); **including *L. kolbeana*; ***including the simple leaf form (Van Wyk, Liu and Magee 4210).

Characters and character states:
1. Habit: woody shrub=0; perennial herb=1. Fig. 1.
2. Root number: few (less than 8 per rhizome, often thick)=0; many (more than 10 per rhizome, usually thin)=1. Fig. 2.
3. Root xylem (fibrous core): not or only slightly fibrous=0; highly fibrous=1. Fig. 2.
4. Root oil ducts: in 2 or 3 rings=0; up to 4 rings=1; in 5 or 6 rings=2. Fig. 2.
5. Root — somewhat lignified cells in secondary phloem: absent=0; present=1. Fig. 2.
6. Leaf persistence: not proteranthous (leaves present at time of fruiting)=0; proteranthous (leaves withered at time of fruiting)=1. Fig. 1.
7. Leaf type: multisect=0; trifid to unifoliate=1. Fig. 3.
8. Leaf margin: teeth unevenly spaced=0; teeth evenly spaced=1. Fig. 4.
9. Marginal teeth: serrate (teeth directed towards the apex)=0; dentate (teeth directed outwards)=1. Fig. 4.
10. Leaf margin: setae absent=0; setae present=1. Fig. 4.
11. Leaf lamina: mesophyll compact=0; mesophyll loosely arranged=1. Fig. 5.
12. Petiole epidermal cells: without minute striae=0; with minute striae=1.
13. Leaf pubescence: glabrous=0; pubescent=1. Fig. 5.
14. Number of rays: few (less than 7)=0; several (more than 7)=1.
15. Length of rays within an umbel: not markedly unequal=0; markedly unequal=1.
16. Flower colour: yellow=0; white=1. Fig. 6.
17. Fruit shape (dorsal view): ovoid=0; oblong=1. Fig. 7.
18. Fruit shape (transverse section): not dorsally compressed=0; dorsally compressed=1. Figs. 7 and 8.
19. Fruit symmetry: homomorphic and heteromorphic=0; homomorphic only=1. Figs. 7 and 8.
20. Fruit ribs: ribs not prominent=0; ribs prominent=1. Figs. 7 and 8.
21. Fruit rib duct shape: round to oval=0; periclinally elongated=1. Figs. 7 and 8.
22. Concentric rings of cells around rib oil ducts: absent=0; present=1.
23. Fruit rib vascular bundles: close together beneath each rib duct=0; widely separated (towards margins of rib ducts)=1. Fig. 7.
24. Pericarp lignification: absent or in commissure only=0; invariably present, extends beyond the commissure=1.
25. Endosperm shape at commissure: slightly concave=0; deeply concave to grooved=1. Figs. 7 and 8.

2. Materials and methods

2.1. Field, herbarium and morphological studies

Field work over a period of several years was necessary to collect complete material of all *Lichtensteinia* species and its regional forms, including herbarium voucher specimens, preserved material for anatomical studies and leaves for DNA extraction (Tables 1 and 2). The opportunity to study plant populations in the field has made an important contribution towards understanding species delimitations. Morphological characters and characters states (see Table 3) were studied *in situ* and by examining these herbarium voucher specimens. Specimens from the following herbaria were also studied (abbreviated according to Holmgren et al., 1990): BM, BOL, GRA, JRAU, K, NBG, P, PRE, S and UPS.

2.2. Anatomical studies

Details of the material used in studying root, leaf, flower and fruit anatomy are provided in Table 2. Suitable portions were embedded in glycol methacrylate according to the method of Feder and O'Brien (1968) and stained using the periodic acid-Schiff/toluidine blue (PAS/TB) method. Slides are housed at JRAU. Additional sections of roots were cut and some were tested with iodine solution (IKI) for the presence of starch. The phloroglucin test for lignin was also performed on other root sections.

2.3. DNA sequencing

Nineteen accessions of *Lichtensteinia* and outgroups were examined for nuclear ribosomal DNA internal transcribed spacer (ITS) and chloroplast DNA (cpDNA) trnQ-5'trnK (hereafter, called trnQ-trnK) sequence variation (Table 3). This cpDNA region includes two intergenic spacers (trnQ-5'rps16 and 3'rps16-5'trnK) and the rps16 intron. These spacers are among the most variable loci useful for molecular phylogenetic study at low taxonomic levels (Shaw et al., 2007). ITS sequences from 14 accessions were obtained specifically for this study, as data for five accessions were previously published (Calviño et al., 2006). Similarly, sequence data for the cpDNA region were available for six accessions through previous studies (Calviño et al., 2006; Calviño and Downie, 2007). Total genomic DNAs were extracted from herbarium specimens or field-collected materials using a DNeasy Plant Mini Kit (Qiagen, Valencia, California, USA). The strategies used to obtain these ITS and cpDNA sequence data are presented elsewhere (Downie and Katz-Downie, 1996, 1999; Calviño et al., 2006; Calviño and Downie, 2007). Simultaneous consideration of both DNA strands across the entire ITS or cpDNA region permitted unambiguous base determination in all taxa.

2.4. Phylogenetic analyses

The morphological and anatomical characters and character states used in the cladistic analysis and the matrix of scored characters are provided in Table 1. The phylogenetic trees were rooted with *Dracosciadium italae* Hilliard and B.L. Burtt. *An-*

Table 2
Material and voucher specimens of *Lichtensteinia* species used for anatomical studies

Species	Voucher specimens	Plant parts studied
L. crassijuga	Esterhuysen 17960 (BOL)	Lamina
	Thompson 3663 (NBG, PRE)	Fruit
	Van Wyk, Boatwright, Magee and Le Roux 4184 (JRAU)	Root
L. globosa	Esterhuysen 17960 (PRE)	Immature fruit
	Magee and Boatwright 33 (JRAU)	Root, lamina, petiole, immature fruit
	Van Wyk and Tilney 4107 (JRAU)	Lamina, flower, immature fruit, almost mature fruit
L. interrupta	Fourcade 5970 (PRE)	Immature fruit
	Hugo 42 (PRE)	Fruit
	Mogg 11851 (PRE)	Lamina
	Montgomery 338 (NBG)	Fruit
	Pegler 891 (PRE)	Fruit
	Tilney 258 (JRAU)	Lamina, petiole
	B-E and M van Wyk Van Wyk 1945 (JRAU)	Lamina
	Winter 131 (JRAU)	Root, petiole
	Winter 161 (JRAU)	Root, lamina, petiole (seedling and mature), fruit
L. lacera	Boucher 1818 (PRE)	Fruit
	Compton 8489 (NBG)	Immature fruit
	Compton 10608 (PRE)	Immature fruit
	Magee and Boatwright 26 (JRAU)	Flower
	Van Rensburg 2138 (PRE)	Lamina
	Van Wyk and Plunkett 4098 (JRAU)	Immature fruit
	Van Wyk 4098 (JRAU)	Root, lamina, petiole
	Winter 82 (JRAU)	Flower, immature fruit, fruit
	Winter 98 (JRAU)	Immature fruit
L. latifolia	Acocks 20337 (PRE)	Fruit
	Manning s.n. (NBG 759103)	Fruit
	Rourke 1698 (NBG)	Flower, immature fruit
	Van Wyk 3568 (JRAU)	Root, lamina, petiole, fruit
	Winter 163 (JRAU)	Root, lamina, petiole
L. obscura	Purcell 464 (NBG)	Fruit
	Salter 8991 (NBG, PRE)	Flower, immature fruit, fruit
	Van Wyk 3523 (JRAU)	Root, lamina, petiole
	Van Wyk and Tilney 4104 (JRAU)	Root, lamina, petiole, immature fruit
L. trifida	Compton 22683 (NBG)	Fruit
	Esterhuysen 18455 (PRE)	Almost mature fruit
	Van Wyk and Tilney 4106 (JRAU)	Lamina, immature fruit
	Van Wyk and Tilney 4164 (JRAU)	Flower, immature fruit
	Winter 155 (JRAU)	Petiole
	Winter 177 (JRAU)	Root, lamina, petiole
L. trifida-simple leaf	Van Wyk, Liu and Magee 4210 (JRAU)	Root, flower, immature fruit

nesorhiza macrocarpa Eckl. and Zeyh. was included as an additional outgroup. *Dracosciadium* Hilliard and B.L. Burtt is a member of tribe Heteromorpheae and is closely related to *Lichtensteinia* (Downie and Katz-Downie, 1999; Calviño et al., 2006). Previous molecular phylogenetic studies have reported a putative sister group relationship between *Lichtensteinia* and the *Annesorhiza* clade plus all other members of Apiaceae subfamily Apioideae (Calviño et al., 2006; Calviño and Downie, 2007). The monotypic Namibian endemic genus

Table 3
Accessions of *Lichtensteinia* and outgroups examined for chloroplast DNA trnQ-5'trnK and nuclear ribosomal DNA ITS sequence variation

Taxon	DNA accession no.	Source and voucher information	GenBank accession numbers
Annesorhiza macrocarpa Eckl. and Zeyh.	2454	South Africa, Noordhoek Village Commonage, near beach, 13 Jan. 2003, Downie 2454 (ILL)	cpDNA: DQ832401, AY838409, DQ832488 ITS: DQ368834
Dracosciadium italae Hilliard and B.L. Burtt	3087	South Africa, Mpumalanga, La Belle Esperance Farm, Luneburg, 3 Mar. 1994, Ngwenya and Singh 1279 (PRE)	cpDNA: EU434657, EU434652 ITS: EU434670
Lichtensteinia crassijuga	3079	South Africa, Nieuwoudt Pass, 26 Nov. 2005, Van Wyk, Boatwright, Magee and Le Roux 4184 (JRAU)	cpDNA: EU434658 ITS: EU434671
L. globosa	2462	South Africa, Elandskloof Pass, upper part of valley, near Middelberg, S of Clanwilliam, 16 Jan. 2003, Downie 2462 (ILL), Van Wyk and Tilney 4107 (JRAU)	cpDNA: DQ832429, AY838429, DQ832517 ITS: DQ368859
L. globosa	3074	South Africa, Pakhuis Pass, 7 Dec. 2004, Magee and Boatwright 33 (JRAU)	cpDNA: EU434659 ITS: EU434672
L. interrupta	3075	South Africa, Kasouga, 19 Dec. 2004, Tilney 258 (JRAU)	cpDNA: EU434660, EU434653 ITS: EU434673
L. interrupta	3089	South Africa, Eastern Cape, Amatole Mountains, Menziesberg, 6 Jan. 1986, Phillipson 1186 (PRE)	cpDNA: EU434661, EU434654 ITS: EU434674
L. interrupta	3091	South Africa, Transkei, Mkambati Nature Reserve, near Daza River, 11 Dec. 1986, Jordaan 995 (PRE)	cpDNA: EU434662, EU434655 ITS: EU434675
L. lacera	2464	South Africa, Silvermine lookout point, 13 Jan. 2003, Downie 2464 (ILL)	cpDNA: DQ832427, AY838427, DQ832515 ITS: EU434676
L. lacera (simple leaf form)	3076	South Africa, Kogelberg, 3 Dec. 2004, Magee and Boatwright 23 (JRAU)	cpDNA: EU434663 ITS: EU434677
L. lacera (simple leaf form)	3077	South Africa, Jonkershoek, 7 Dec. 2004, Magee and Boatwright 29 (JRAU)	cpDNA: EU434664 ITS: EU434678
L. lacera (simple leaf form)	3078	South Africa, Sir Lowry's Pass (narrow leaf), 2 Dec. 2004, Magee and Boatwright 22 (JRAU)	cpDNA: EU434665 ITS: EU434679
L. latifolia	3080	South Africa, Swellendam, 3 Feb. 2006, Van Wyk et al. 4212 (JRAU)	cpDNA: EU434666 ITS: EU434680
L. obscura	2457	South Africa, Olifants River, between Citrusdal and Clanwilliam, 15 Jan. 2003, *Downie 2457* (ILL), Van Wyk and Tilney 4104 (JRAU)	cpDNA: DQ832428, AY838428, DQ832516 ITS: DQ368858
L. obscura	3081	South Africa, Kardouw, Citrusdal, 26 Nov. 2005, Van Wyk et al. 4187 (JRAU)	cpDNA: EU434667 ITS: EU434681
L. obscura	3086	South Africa, Cape Peninsula, Camp Ground, Salter 8991 (PRE)	cpDNA: EU434668, EU434656 ITS: EU434682
L. trifida	2460	South Africa, Middelberg, Piekenierskloof Pass (picnic site), 15 Jan. 2003, Downie 2460 (ILL)	cpDNA: DQ832430, AY838430, DQ832518 ITS: DQ368860
L. trifida	2461	South Africa, Elandskloof Pass, upper part of valley, near Middelberg, S of Clanwilliam, 16 Jan. 2003, Downie 2461 (ILL), Van Wyk and Tilney 4106 (JRAU)	cpDNA: DQ832431, AY838431, DQ832519 ITS: DQ368861
L. trifida (simple leaf form)	3082	South Africa, Teewaterskloof Dam, 28 Jan. 2006, Van Wyk, Liu and Magee 4210 (JRAU)	cpDNA: EU434669 ITS: EU434683

Three GenBank accession numbers for cpDNA represent the trnQ-rps16 intergenic spacer, rps16 intron, and rps16-5'trnK intergenic spacer regions, respectively. Two GenBank numbers for cpDNA represent the trnQ-rps16 intron region (with no rps16 3'exon) and the rps16-5'trnK intergenic spacer region, respectively. A single GenBank number for cpDNA represents the entire trnQ-5'trnK region.

Marlothiella H. Wolff may also be closely related to *Lichtensteinia* based on the shared presence of concentric rings of cells around the large rib oil ducts; therefore, *Marlothiella* was also included. Phylogenetic analyses were performed with PAUP* vers. 4.0b10 (Swofford, 2002) with branch and bound searches and characters treated as unordered and equally weighted. Internal support was assessed using 10,000 bootstrap (BS) replicates (Felsenstein, 1985). The number of additional steps required to force particular taxa into a monophyletic group was examined using the constraint option of PAUP*.

In the molecular analyses, the phylogenetic trees were also rooted with *Dracosciadium italae*. *Annesorhiza macrocarpa* was used as an additional outgroup, as in the analysis of morpholo-gical/anatomical data. However, *Marlothiella gummifera* was not included in this component of the study because DNA sequences are not currently available. The ITS and cpDNA trnQ-trnK data matrices were analyzed separately and combined using maximum parsimony, as implemented by PAUP. Branch and bound searches were conducted for each analysis. Characters were treated as unordered and equally weighted, and gap states were treated as missing data. Bootstrap values were calculated from 10,000 replicate analyses using TBR branch swapping and simple stepwise addition of taxa. The partition homogeneity test of PAUP* was used to examine the extent of conflict between data partitions. This test was carried out with 100 replicate analyses, using the heuristic search option, simple stepwise addition of taxa,

TBR branch swapping, and a maxtrees setting of 5000. Bayesian inference of combined cpDNA trnQ-trnK and ITS data sets was conducted using the program MrBayes version 3.1.1 (Huelsenbeck and Ronquist, 2001). Prior to analysis, Modeltest version 3.5 (Posada and Crandall, 1998) was used to select an evolutionary model of nucleotide substitution that best fits these data, as selected by the Akaike Information Criterion estimator (Posada and Buckley, 2004). The best-fit models selected were K81uf+G

Table 4
Comparison of taxonomically useful characters and character states in *Lichtensteinia* species

Species	L. crassijuga	L. globosa	L. interrupta	L. lacera	L. latifolia	L. obscura	L. trifida
Number of roots							
Few	+	−	−	+	+	−	+
Several in cluster	−	+	+	−	−	+	−
Central root core							
Highly fibrous (with gelatinous fibres)	+	+	+/−	−	−	+	−
Lignified	−	−	+/−	−	−	−	−
Oil ducts in root							
Number of rings	6	2/3	2/3	5/6	2/3/4	3	5
Maximum diameter (μm)	±210	±140	±170	±230	±330	±170	±120
Thick-walled cells (gelatinous fibres) in sec. phloem of root:							
In rings	+	+	+/−	+	−	+	+
Somewhat lignified	−	−	−	+	−	−	+
Leaves proteranthous	+	+	+/−	+	+	+	+
Division of leaf							
Divided (± pinnatisect)	+	+	+	−	−	+	−
trifid to unifoliate	−	−	−	+	+	−	+
Leaf margins							
Serrate	−	+	+	−	−	+	−
Dentate	+	−	−	+	+	−	+
Marginal teeth							
Evenly spaced	−	−	−	+	+	−	+
Teeth with setae	−	+	−	+	−	−	+/−
Lamina							
Mesophyll compact	−	+	+	−	−	+	−
Mesophyll loosely arranged	+	−	−	+	+	−	+
Petiole epidermal cells: with distinct minute striae	−	+	+	−	−	+	−
Pubescence							
At least on margin	+	−	−/+	+	+	+/−	+
Epidermal cells form 'cushions' for hairs	−	−	−	−	−	−	+
Main umbel: number of rays	10	(2)3–4	3–14	7–20	9–20	(2)3–4(10)	6–13
Rays: markedly unequal	+	−	−	+	+	−	+
Flower colour							
White	−	+	+	−	−	−	−
Yellow	+	−	−	+	+	+	+
Style length: elongated	+	−	−	+	+	−	+
Fruit shape							
Dorsally compressed	+	−	−	+	+	−	+
Oblong	+	−	−	+	+	−	+
Ovoid	−	+	+	−	−	+	−
Fruit symmetry							
Homomorphic only	−	+	+	−	−	+	−
Homomorphic or heteromericarpic	+	−	−	+	+	−	+
Fruit surface: with protuberances	−	+	−	−	−	−	−
Fruit ribs: prominent	+	−	−	+	+	−	+
Rib oil duct shape							
Round to oval	+	+	−	+	+	−	+
Periclinally elongated	−	−	+	−	−	+	−
Rib oil duct relative size: dissimilar to one another∗	−	+	−	−	−	−	−
Position of rib vascular bundles relative to one another: widely separated	−	+	+	−	−	+	−
Pericarp lignification: commissure and beyond	+	−	−	+	+	−	+
Endosperm shape at commissure							
Slightly concave	−	−	−	+	+	−	+
Deeply concave to grooved		+	+	−	−	+	−

∗Excluding heteromericarpic condition.

and GTR+I+G for the trnQ-trnK and ITS data partitions, respectively. From different random starting trees, two independent analyses were run for 2 million generations and the trees saved to a file every 100 generations (i.e., a total of 20,000 trees was sampled). Stationarity and convergence search strategies are the same as employed in Calviño and Downie (2007). The combined data matrix was also analyzed using the maximum likelihood method, as also implemented by PAUP*. The results obtained were congruent to those inferred by the Bayesian analysis; hence, they will not be discussed further.

The morphological/anatomical and molecular data matrices were combined for a total evidence analysis using maximum parsimony and Bayesian methods. Search strategies were the same as described for the molecular analysis. In the Bayesian analysis, each of the three data partitions (i.e., cpDNA trnQ-trnK, ITS, morphology/anatomy) was specified its corresponding model of evolution, with the morphology/anatomy partition being treated with the "standard discrete" model. The partition homogeneity test of PAUP* was used to examine the extent of conflict between data partitions, as previously described.

3. Survey of morphological and anatomical characters

A summary of taxonomically useful morphological and anatomical characters and character states is presented in Table 4. In the discussion below, *L. globosa* refers to a new species (Van Wyk and Tilney, in press). Furthermore, *L. kolbeana* L. Bolus and *L. interrupta* are regarded as a single species and the name

L. interrupta is therefore used in a broad sense to include *L. kolbeana*, unless otherwise stated.

3.1. Habit

The typical herbaceous habit of *Lichtensteinia* species is shown in Fig. 1. Plants may have a distinct rhizome or a short to very short rhizome-like axis on which radical leaves are produced each year. The inflorescences are erect and may reach a height of between 0.25 m and 1.5 m depending not only on the species but also on local growing conditions.

3.2. Roots

The rhizomes and roots generally can be divided into two types: several, relatively thin roots (usually 2–4(6) mm in diameter) arising in clusters from the base of old leaves (Fig. 2A) on a usually poorly defined axis, or a few, relatively thick roots (usually at least some being a minimum of 5 mm in diameter) arising from a generally well-developed rhizome (Fig. 2B). Roots of the former type tend to be exceptionally tough due largely to a fibrous core which frequently detaches from the rest of the organ when uprooted. This core is usually less fibrous in the latter type. The thinner roots frequently have numerous short lateral roots arising more or less at right angles. Lateral roots are frequently sparse on the more fleshy type.

Root transverse sections were studied and the main characters illustrated in Fig. 2C–F. Anatomically, the mature
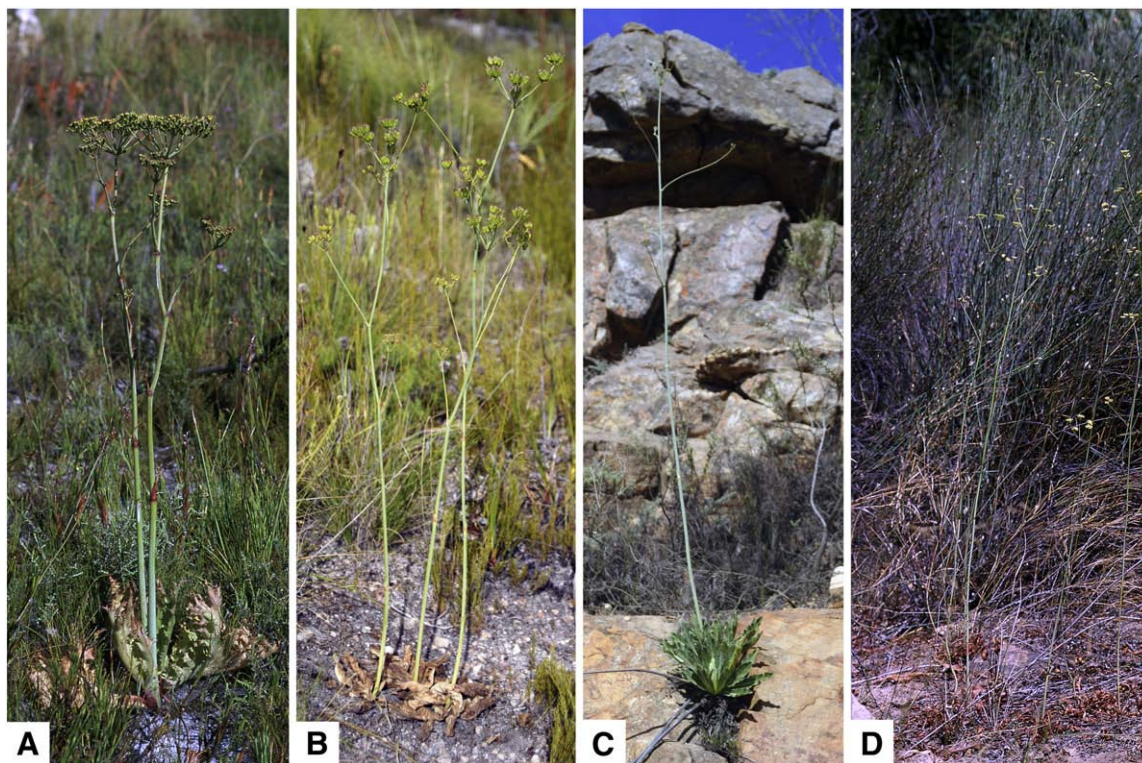

Fig. 1. Habit of *Lichtensteinia* species. (A) *L. lacera* (flowering plant at Noordhoek Pass on the Cape Peninsula; height ca. 0.65 m); (B) *L. trifida* – simple leaf form (fruiting plant at Teewaterskloof Dam near Villiersdorp; note that the leaves are proteranthous – they shrivel during or after flowering; height ca. 0.62 m); (C) *L. trifida* — trifid form (starting to flower in Middelberg Pass near Citrusdal; height ca. 1.1 m); (D) *L. obscura* (flowering near Citrusdal — note that the leaves have withered; height ca. 1.2 m). All photographs taken by B-E. Van Wyk.
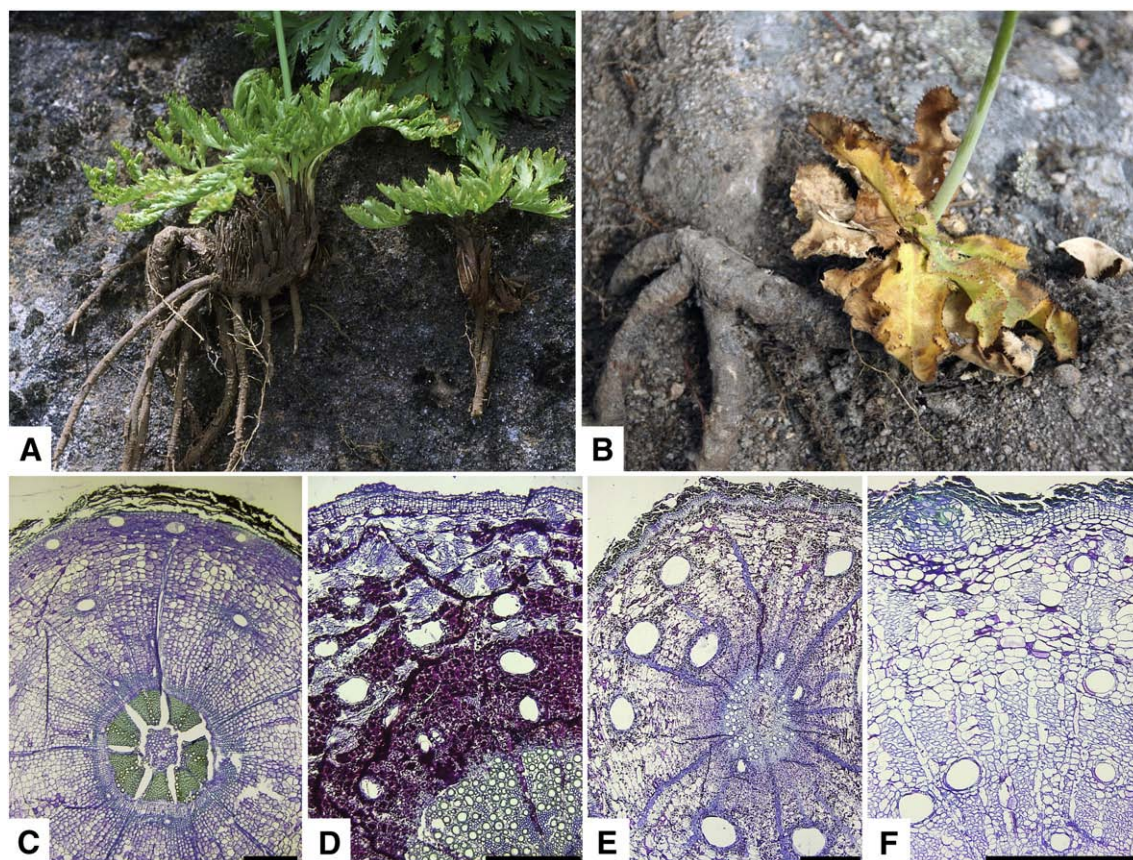
Fig. 2. Root morphology and anatomy in *Lichtensteinia* species. (A) plant of *L. globosa* (Van Wyk and Tilney 4107), showing a cluster of thin, slightly fleshy roots arising from the rhizome (rhizome length ca. 60 mm); (B) plant of *L. trifida* (simple-leaved form, Teewaterskloof population, Van Wyk, Liu and Magee 4210) showing a few thick and fleshy roots (visible part of rhizome ca. 100 mm long); (C) transverse section through a root of *L. interrupta* (Winter 161) showing the highly lignified fibrous core and lack of rings of thickened cells in the secondary phloem; (D) close up of transverse section through a root of *L. crassijuga* (Van Wyk et al. 4184) showing the many oil ducts and fibres with gelatinous cell walls in the secondary phloem; (E) transverse section through a root of *L. latifolia* (Van Wyk 3568) showing the non-fibrous core, a lack of fibres in the secondary phloem and large oil ducts; (F) close up of transverse section through a root of *L. trifida* (Winter 177) showing the abundance of thickened lignified cells in the secondary phloem and the initiation of the periderm from the primary phloem. Scale bars=0.5 mm. Photographs A and B taken by B-E. Van Wyk.

root has a stem-like appearance with a pith and consists mainly of secondary vascular tissue. The xylem is generally weakly developed and, proportionally, the secondary phloem is very well developed. There are clear medullary rays. The secondary phloem in both specimens of *L. obscura* is conspicuously stratified. There are rings of thin-walled storage parenchyma cells with starch grains alternating with zones of non-storage, laterally-compressed cells (fibres) with thickened walls. These thickened cell walls appear to have a gelatinous layer (G-layer) and show little or no lignification. Similar rings, but somewhat less regular, were observed in *L. interrupta* (Winter 131), *L. lacera* Cham. and Schlechtd. and *L. trifida* Cham. and Schlechtd., and markedly less regular in *L. crassijuga* E. Mey. ex Sond. (Table 4). A single ring of cells, not markedly thickened, is visible in *L. globosa*, and isolated small areas of essentially unthickened cells are present in *L. latifolia* Eckl. and Zeyh. *Lichtensteinia interrupta* (Winter 161) (Fig. 2C) also appears to lack rings of thickened cells in the secondary phloem. *Lichtensteinia crassijuga* (Fig. 2D) and *L. trifida* (Fig. 2F) have the greatest number of thickened cells, and only in the latter species and in *L. lacera* do they show some lignification. The

thickened cells no doubt contribute to the tough nature of the roots. The tracheary elements of very young roots usually appear to be accompanied by much xylem parenchyma. When secondary thickening occurs, large numbers of fibres may form thus producing the fibrous core characteristic of the roots of certain *Lichtensteinia* species (Fig. 2C; Table 4). Only in one specimen (*L. interrupta,* Winter 161) were these cells highly lignified (Fig. 2C). In *L. crassijuga*, *L. globosa*, *L. interrupta* (Winter 131) and *L. obscura* the fibres have a cell wall that is essentially gelatinous (contains a G-layer) — only slight lignification is occasionally evidenced by a very pale pink colour with the phloroglucin test. No green colour with the Schiff's/toluidine blue method was visible in these species. Similar fibres were found in *L. lacera* but in very small numbers. Oil ducts occur in the secondary phloem and vary in size, number of concentric rings and number per ring (Fig. 2C–F; Table 4). They are absent from the xylem. Generally the species with the fewest (and largest) roots have the greatest number of rings of oil ducts (Table 4). The diameter of the oil ducts usually does not exceed ±170 μm except in *L. crassijuga*, *L. lacera* and *L. latifolia* (Van Wyk 3568) where diameters of

±210, ±230 and ±330 μm respectively were observed. The diameter of the oil ducts in the other specimen of *L. latifolia* (Winter 163) was, however, ±140 μm. Starch grains are generally present in large numbers in the root cells with the greatest concentrations usually being associated with the oil ducts. Starch-containing cells are particularly abundant and
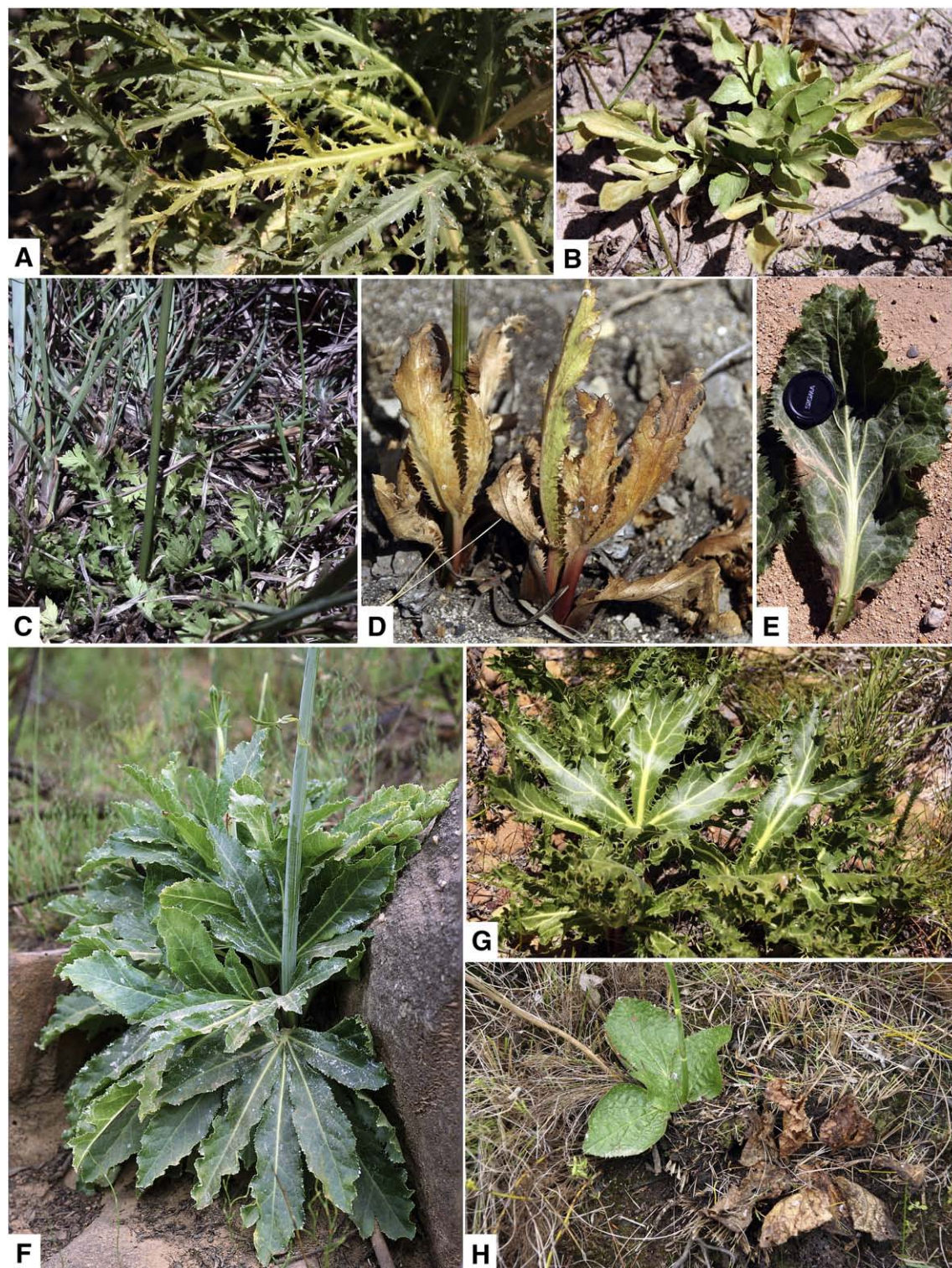

Fig. 3. Leaf morphology of *Lichtensteinia* species. (A) lacerate leaves of *L. crassijuga* at Nieuwoudt Pass near Citrusdal (0.2×); (B) pinnately compound leaves of *L. obscura* at the Baths near Citrusdal (0.3×); (C) pinnately compound leaves of *L. interrupta* (=*L. kolbeana*) at Kasouga (0.9×); (D) trifid leaves of *L. lacera* at Sir Lowry's Pass (0.21×); (E) entire leaves of *L. lacera* at Jonkershoek (0.15×); (F) leaves of *L. trifida* at Middelberg Pass near Citrusdal (0.16×); (G) leaves of *L. lacera* at Camps Bay, Cape Town (0.15×); (H) leaves of *L. latifolia* at Swellendam (0.23×). Photographs A, B, D-H taken by B-E. Van Wyk; photograph C taken by P.V. Dickason.

very prominent in *L. globosa*. No crystals were observed in any of the species. The periderm, in at least some species, is deep-forming being initiated from the primary phloem (Fig. 2F).

The roots of all species except *L. crassijuga* thus fall into one of two main types: (1) few and relatively thick roots, lacking a central core of fibres and many rings of oil ducts (*L. lacera*, *L. latifolia* and *L. trifida*); and (2) several, relatively thin roots with a central core of fibres and with few rings of oil ducts (*L. globosa*, *L. interrupta* and *L. obscura*). *Lichtensteinia crassijuga*, although fitting more closely with the former type, has a fibrous core as in the second type.

3.3. Leaves

The leaves are arranged in a basal rosette (Fig. 1). A characteristic feature of *Lichtensteinia* is the proteranthous leaves. Only when the leaves start to shrivel and die is a flowering stem produced. The only exception is the eastern form of *L. interrupta*, currently known as *L. kolbeana*, which may have flowers and leaves on the same plant. However, in this form the leaves generally start dying before the fruits are fully mature. The remains of old leaf bases are frequently visible as fibres and may often form a dense mass. The leaves are petiolate with the petioles being broad at the base. They vary from simple to compound (Fig. 3) and this variation may be encountered even in a single plant. However, the leaves of all species are generally based, to a greater or lesser degree, on a trifoliate and palmate pattern which is usually evident in at least one leaf of a plant. Leaf segments may, nevertheless, be either simple or pinnatisect. Although the leaf shape and size within a species may vary quite considerably, these characters are often diagnostic (Fig. 3). The leaf margins are also taxonomically useful (Fig. 4). Despite some variation, two main types, viz. serrate and dentate, can be distinguished. As summarized in Table 4, *L. interrupta, L. globosa* and *L. obscura* have more or less irregular, margin serrations while the other species (*L. lacera*, *L. latifolia* and *L. trifida*) have evenly spaced dentate margins. Leaf segments of *L. crassijuga* and *L. latifolia* often have short, subulate teeth/serrations being uneven in the former and fairly even in the latter; those of *L. globosa* have protracted and fairly even serrations often ending in setae; those of *L. lacera* have greatly protracted and uneven teeth, usually
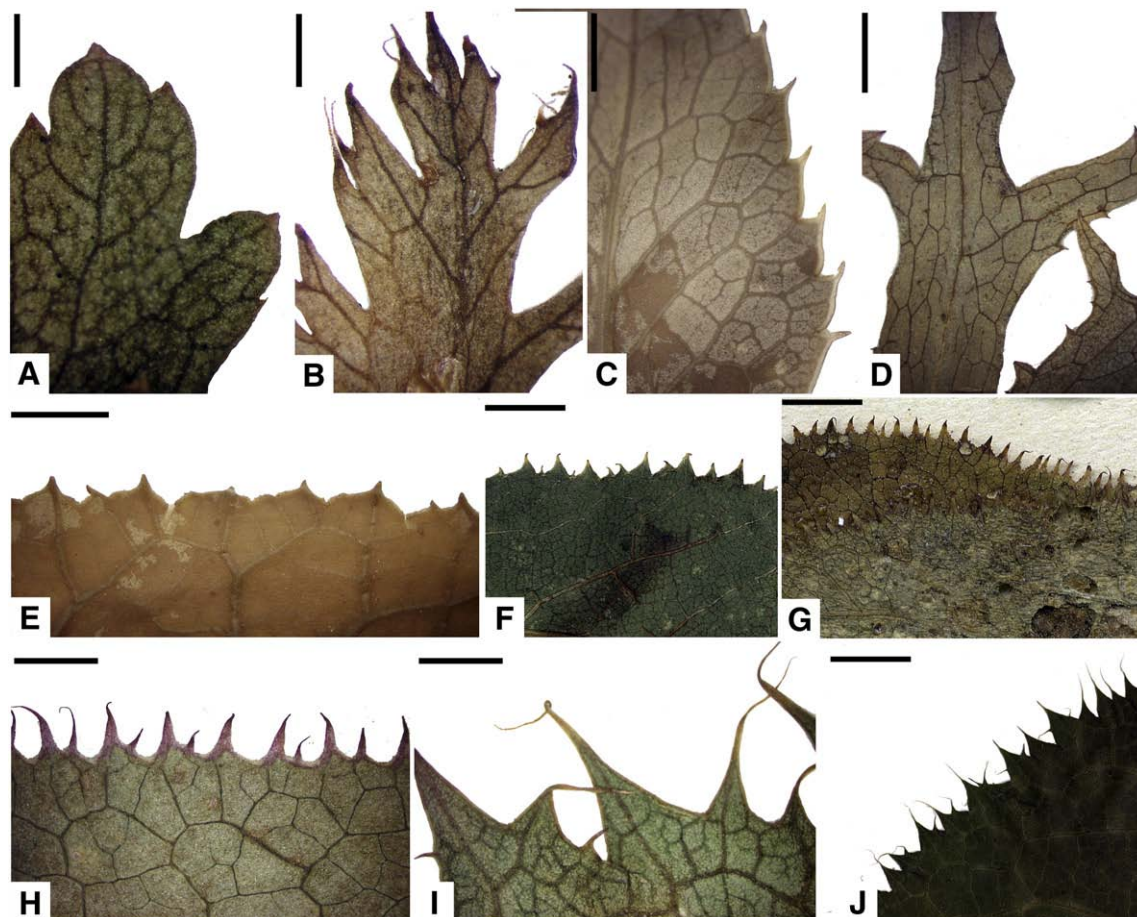

Fig. 4. Leaf margins of *Lichtensteinia* species. (A) coarsely serrate (*L. interrupta*, Winter 161); (B) coarsely serrate with acute teeth dentate (*L. globosa*, Magee and Boatwright 33); (C) serrate with somewhat mucronate teeth (*L. obscura*, Van Wyk et al. 4187); (D) sparsely serrate-dentate with teeth pointing sideways (*L. crassijuga*, Esterhuysen 17960); (E) coarsely dentate with obtuse teeth (*L. latifolia*, Van Wyk 3568); (F) dentate (*L. trifida*, Van Wyk and Tilney 4106); (G) denticulate (*L. trifida* – simple-leaved form, Marloth 8149); (H) dentate with small, regularly spaced, narrowly triangular teeth with short, hair-like tips (*L. lacera*, Magee and Boatwright 22); (I) irregular, coarsely dentate with triangular teeth ending in long, drawn out, hair-like tips (*L. lacera*, Van Wyk 4098); (J) coarsely dentate with triangular teeth ending in drawn out, hair-like tips (*L. lacera*, Van Wyk 4197). Scale bars: A, B = 1 mm; C = 2 mm; D, E, H, I = 4 mm; F, G, J = 10 mm.

ending in distinct setae; and those of *L. trifida* may have shortly protracted teeth which are more or less evenly spaced, with setae nearly always very short or sometimes absent. The size and shape of marginal teeth and the presence and length of setae are of considerable diagnostic value, especially to distinguish among *L. latifolia*, *L. trifida*, *L. lacera* and *L. crassijuga*.

Transverse sections of petioles revealed very little difference among the species. The vascular bundles are arranged in crescents with usually about seven to nine bundles in the smaller-leaved species (Fig. 5A), but up to about 17 or more in the larger-leaved species. Many vascular bundles have a small amount of primary phloem situated adaxially. Epidermal cells with minute but distinct striae were observed in *L. globosa*, *L. interrupta*, *L. obscura* and, to a lesser degree, in one specimen of *L. trifida*. Sub-epidermal strands of collenchyma are present, being distinctly lamellar in most species. Oil ducts are nearly always found to the outside and inside of each vascular bundle. Crystals are present in at least some of the species. Nearly all the petioles examined have central cavities. These were found to be present even in a seedling of *L. interrupta* (*L. kolbeana* form).

Hairs were observed on the leaves, in varying numbers, in at least some specimens of all the species except *L. globosa*. They are often present on both abaxial and adaxial surfaces occurring along almost all the veins, even very small ones, and the leaf margins, but may be very difficult to distinguish in dry material without a microscope. Hairs appear to be most consistently present in *L. trifida* (Fig. 5B,E) where they are also usually larger than those of the other species. Most of the specimens of *L. interrupta* (including *L. kolbeana*) appear to be glabrous. Hairs, when present on the leaves, are unicellular. In *L. trifida* the epidermal cells surrounding the hair bases of the lamina tend to form cushions. All species have amphistomatic leaves (Fig. 5C). The epidermal cells in a single specimen may vary considerably in size with usually the largest cells being associated with the veins. The outer periclinal cell walls of the epidermal cells vary in degree of cutinisation, being the most highly cutinised in *L. globosa*. The mesophyll is nearly always distinctly differentiated into palisade and spongy parenchyma with the palisade parenchyma being 1 or 2(3)-layered. The cells of the spongy mesophyll of *L. globosa*, *L. interrupta* (Fig. 5C) and *L. obscura* tend to be more compact than in *L. crassijuga*, *L. lacera*, *L. latifolia* (Fig. 5D) and *L. trifida*. Oil ducts are present above and below the vascular bundles of the larger veins. The vascular bundles of these veins are often also accompanied by conspicuous lamellar collenchyma. Crystals occur in at least some of the species.
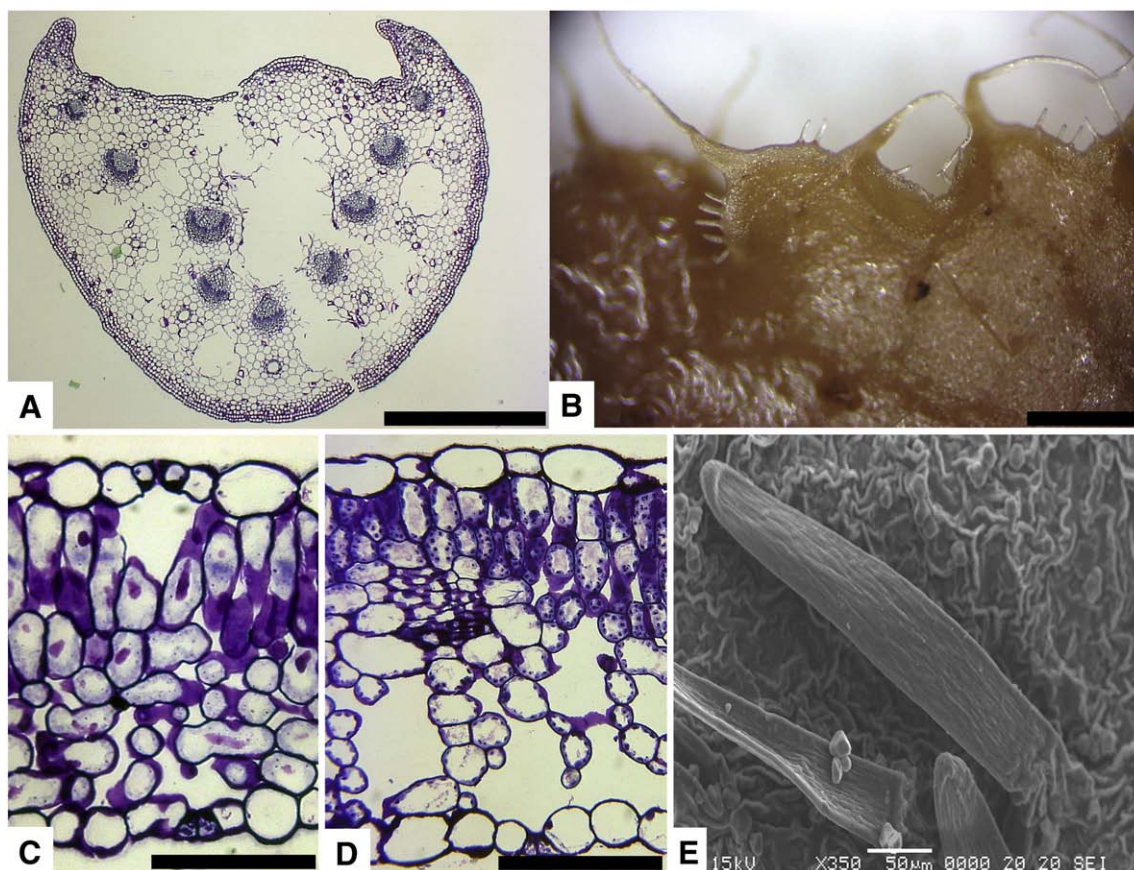

Fig. 5. Anatomical details of the petiole and leaf lamina of *Lichtensteinia* species. (A) transverse section of the petiole of *L. globosa* (Magee and Boatwright 33) showing the typical structure of all the species; (B) setae and short hairs on the marginal teeth of *L. trifida* (Winter 177); (C) transverse section of the lamina of *L. interrupta* (Winter 161) showing the compact arrangement of the chlorenchyma; (D) transverse section of the lamina of *L. latifolia* (Winter 163) showing the loosely arranged chlorenchyma cells; (E) Scanning electron microscope (SEM) photograph of hairs on the abaxial leaf lamina of *L. trifida* (Van Wyk and Tilney 4106). Scale bars: A, B = 1 mm; C, D = 0.07 mm.

The cotyledons are rounded, the so-called R type of Cerceau-Larrival (1962), and toothed, which Burtt (1991) pointed out may be unusual. Burtt (1991) mentioned that the first foliage leaf in *Lichtensteinia* is palmate as in a wide range of other Apiaceae that eventually produce pinnatisect sterile adult leaves.

3.4. Flowers

Flowers (Fig. 6) are borne in large compound umbels. In most species there are between about eight to 11 rays in the main umbel but far fewer are usually found in *L. globosa* and *L. obscura* (Table 4). The rays are markedly unequal in length in *L. crassijuga*, *L. trifida*, *L. latifolia* and *L. lacera*. The basic structure of the flower of *Lichtensteinia* is typical of the family. The sepals tend to be essentially ovate-acute or ovate-acuminate (Fig. 6A,C,D). The petals may be yellow or white (Fig. 6A,B) and they have very conspicuous oil ducts (visible in Fig. 6C,F). The styles and/or stylopodium are prominent (Fig. 6A,D). The two carpels making up each ovary are nearly always similar macroscopically but sometimes a distinct difference in the size of the rib oil ducts is evident between the median and lateral ribs of the two mericarps making up a single fruit thus giving a heteromorphic (heteromericarpic) appearance (discussed later). Enormous oil ducts are present in the ovary but vittae are absent. Crystals are also visible.

In the present study, serial cross sections through a flower of *L. lacera* (Fig. 6F) showed that the inflexed petal apices are joined by a well-developed septum (Fig. 6E) to the basal portion of the petals, thus forming a tube extending from above the styles, down their length to the stylopodium. The close association between the tissues of the petals and those of the anthers is also visible. This appears to be a similar set-up to that recorded by Bell (1971) for *Eryngium* L.

3.5. Fruits

The fruit (Fig. 7) is crowned with the persistent calyx teeth, styles and stylopodium. The ribs are fairly conspicuous to inconspicuous and the fruits are homomericarpic (Fig. 7B) or slightly heteromericarpic (Fig. 7D) (essentially only clearly evident in transverse section). It is interesting that not all samples of the species with heteromericarpic fruits showed this feature — some fruits appeared to be homomericarpic. There are two main fruit types: shape ovoid, terete, homomericarpic, stylopodium persistent and prominent (*L. globosa*, *L. interrupta* and *L. obscura*) (Fig. 7A,B); and shape oblong, dorsally compressed, and homomericarpic or heteromericarpic (*L. crassijuga, L. lacera, L. latifolia* and *L. trifida*) (Fig. 7C, D). In the latter group, the styles, and usually also the stylopodia, are elongated. In the other group, the stylopodia are well-developed but the styles are usually shorter.
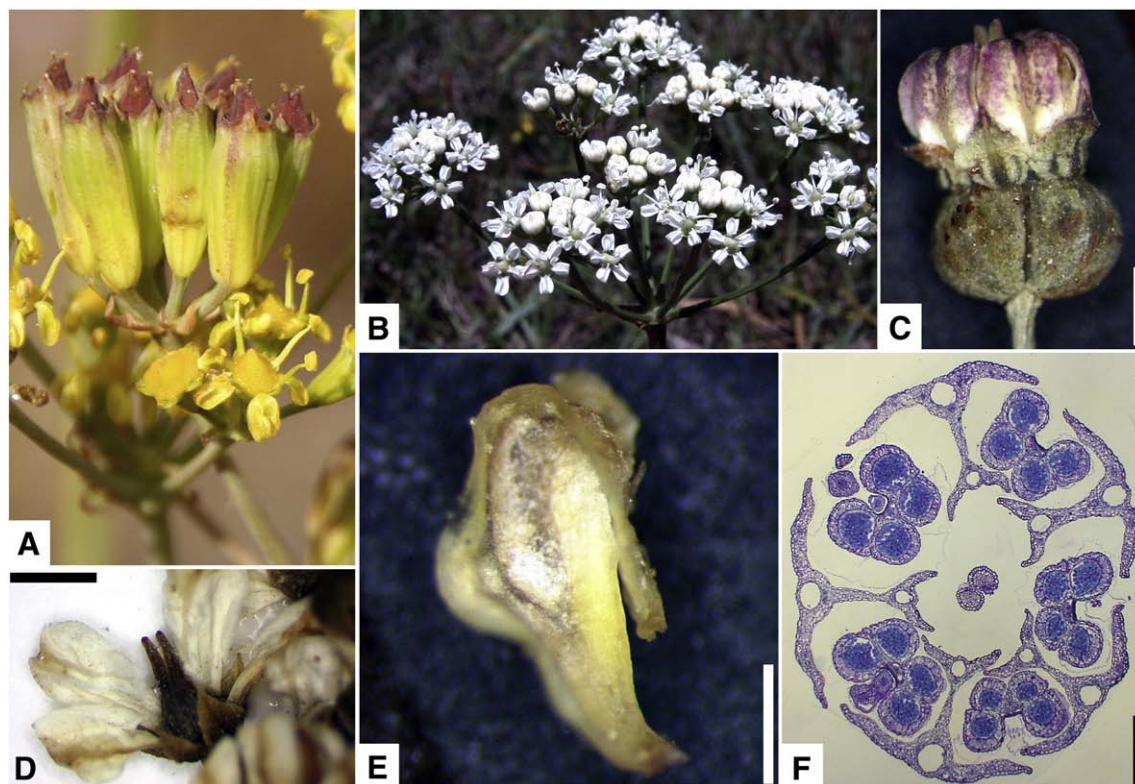
Fig. 6. Flowers of *Lichtensteinia* species. (A) flowers (and green fruit) of *L. trifida* – simple leaf form (Van Wyk, Liu and Magee 4210) showing the prominent styles and yellow flower colour (2.7×); (B) flowers of *L. interrupta* (=*L. kolbeana*) (Tilney 258) showing the white flower colour (1.3×); (C) flowers of *L. globosa* (Magee and Boatwright 33) showing the inflexed petals with broad median oil ducts; (D) flower of *L. globosa* (Van Wyk and Tilney 4107) showing the inflexed petals, white flower colour and prominent stylopodium; (E) single petal of *L. interrupta* (Tilney 258) showing the inflexed tip and septum; (F) section of flower of *L. lacera* (Magee and Boatwright 26), showing the inflexed petals and septa. Photograph A taken by B-E. Van Wyk and B by P.V. Dickason. Scale bars: C, D=1 mm; E, F=0.5 mm.

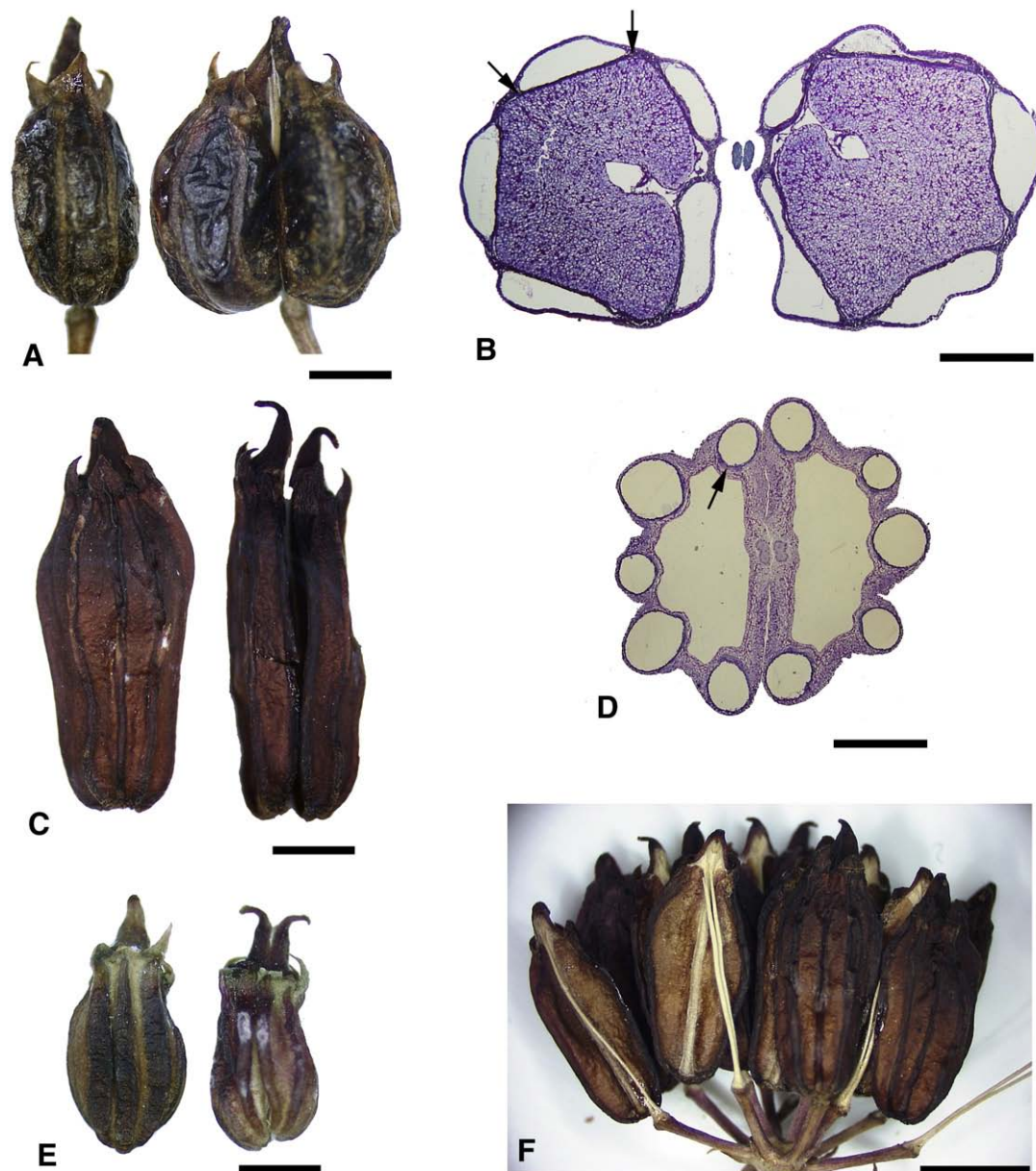Fig. 7. Fruit structure in the genus *Lichtensteinia*. Fruits in dorsal and/or ventral view (A left, C left, F), lateral view (A right, C right, E) and transverse section (B, D), showing the ovoid (A, B) to strongly flattened (C, D) mericarps. (A) fruit of *L. obscura* (Purcell 464) showing the persistent calyx; (B) transverse section of the fruit of *L. interrupta* (=*L. kolbeana*) (Pegler 891); (C) fruit of *L. lacera* (Boucher 1818); (D) transverse section of the fruit of *L. trifida* (Van Wyk and Tilney 4164) – note that this fruit is distinctly heteromorphous; (E) fruit of *L. latifolia* (Acocks 20337) – note the long stylopodium and styles; (F) fruits of *L. lacera* (Boucher 1818) – note the bipartite carpophores. Arrows indicate position of vascular bundles. Scale bars: *A, C, E*=2 mm; *B, D*=1 mm; *F*=4 mm.

Lichtensteinia fruits may have fairly conspicuous (*L. crassijuga*, *L. lacera*, *L. latifolia* and *L. trifida*) or somewhat inconspicuous ribs (*L. globosa*, *L. interrupta* and *L. obscura*), but the rib oil ducts are characteristically exceptionally large (Fig. 7B,D). The prominent styles and/or stylopodia persist and form distinctive features of the fruits. The ventral bundles are opposite and form two well-developed, bipartite carpophores (Fig. 7F). In *L. globosa*, *L. interrupta* and *L. obscura* there are two vascular bundles associated with each rib oil duct which are usually widely spaced so that they tend to be somewhat laterally positioned in relation to the rib oil ducts (indicated by arrows in Fig. 7B), whereas in the other species (*L. crassijuga*, *L. lacera*,

L. latifolia and *L. trifida*) they are close or fairly close together and are situated directly below each rib oil duct (Fig. 7D).

There is considerable variation in the shape of the mericarps and the size and shape of the rib oil ducts (Fig. 8). The mericarps are typically isodiametric in *L. globosa*, *L. interrupta* and *L. obscura* (Fig. 8A−D), while they are dorsally compressed in *L. latifolia* (Fig. 8E) and *L. crassijuga* (Fig. 8G) and markedly so in *L. lacera* (Fig. 8H) and *L. trifida* (Fig. 8F). The rib oil ducts are rounded to somewhat oval in some species (*L. crassijuga*, *L. globosa*, *L. lacera*, *L. latifolia* and *L. trifida*) but markedly periclinally elongated in others (*L. interrupta* and *L. obscura*). In all three specimens studied of *L. globosa*, there is a marked
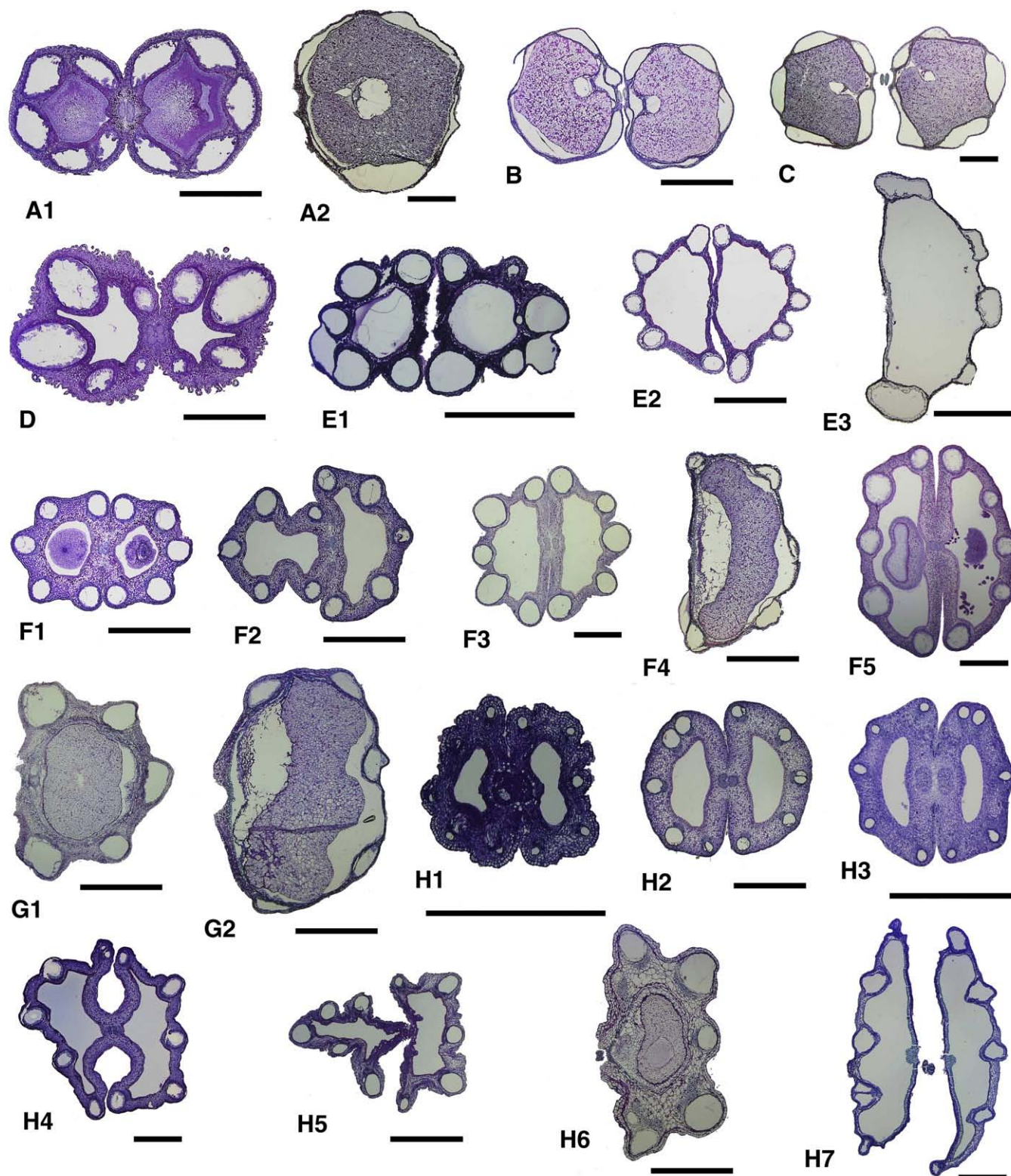
Fig. 8. Transverse sections of immature and mature fruits of all the species of *Lichtensteinia* to show differences in shape, symmetry, rib oil ducts and surface sculpturing. (A1) *L. obscura*, immature fruit (Van Wyk and Tilney 4104); (A2) *L. obscura*, mature fruit (Purcell 464); (B) *L. interrupta*, mature fruit (Hugo 42); (C) *L. interrupta* (=*L. kolbeana*), mature fruit (Pegler 891); (D) *L. globosa*, immature fruit (Van Wyk and Tilney *4107*); (E1) *L. latifolia*, immature fruit (Rourke 1698); (E2) *L. latifolia*, mature fruit (Acocks 20377); (E3) *L. latifolia*, mature fruit (Manning s.n. NBG 759103); (F1) *L. trifida*, immature fruit (Van Wyk and Tilney 4106); (F2) *L. trifida*, immature fruit (Van Wyk and Tilney 4106); (F3) *L. trifida*, immature fruit (Van Wyk and Tilney *4164*) (F4) *L. trifida*, mature fruit (Compton 22683); (F5) *L. trifida* (simple leaf form, Teewaterskloof population), immature fruit (Van Wyk, Liu and Magee *4210*); (G1) *L. crassijuga*, immature fruit (Thompson 3663); (G2) *L. crassijuga*, mature fruit (*Thompson 3663*); (H1) *L. lacera*, young fruit (Compton 8489), (H2) *L. lacera*, immature fruit (Van Wyk and Plunkett 4098); (H3) *L. lacera*, immature fruit (Winter 98); (H4) *L. lacera*, immature fruit (Winter 82); H5, *L. lacera*, immature fruit (Compton 10608); (H6) *L. lacera*, mature fruit (Boucher 1818); (H7) *L. lacera*, mature fruit (Boucher 1818). Scale bars=1 mm.

difference in the size of the rib oil ducts, with the commissural ones usually being the smallest. In the other species the size was more or less uniform.

Relatively few of the median transverse sections through the dorsally compressed fruits (Fig. 8E–H) showed seeds. This seems to be due to the seeds often only developing partially or not at all (as shown by longitudinal sections, not illustrated here). Where seeds were observed, those of *L. crassijuga*, *L. lacera* and *L. trifida* were slightly concave at the commissural side, whereas those of *L. interrupta* and *L. obscura* were deeply concave to grooved.

Lignification of the commissural region (Fig. 9A), often extending further and including cells of the endocarp and sometimes the mesocarp, was observed in specimens of *L. crassijuga*, *L. lacera*, *L. latifolia* and *L. trifida*. The commissure is narrow even in those fruits that are markedly compressed. Crystals are evident in the mesocarp and occur all around the seed but tend to be more concentrated in the commissural area (Fig. 9B). In the mesocarp they are frequently concentrated adjacent to the epidermis and/or endocarp. In the commissural region their distribution suggests that they play a role in facilitating the separation of the ventral vascular bundles and the commissure (Fig. 9B). *Lichtensteinia globosa* was the only species found to have surface features (protuberances) on the mericarps (Fig. 9E) apart from minute striae or papillae on the epidermal cells and/or cuticles of most species. These protuber-

ances are concentrated in the valleculae and are highly distinctive for this species.

The lumen of each rib oil duct is surrounded by a few layers of irregular epithelial cells. This is in marked contrast to the single layer of regular cells present in almost all of the large number of genera that have been studied (e.g. Liu, 2004). The only exception is the monotypic genus *Marlothiella* (Liu et al., 2007), in which we have observed the identical feature (Fig. 9D).

The fruits of *Lichtensteinia* species fall into two main types depending on the shape, compression, relative appearance of the styles and stylopodia, as well as the mericarps comprising the fruit (homomericarpic or heteromericarpic), the degree of development of the ribs, the position of the vascular tissue in relation to the rib oil ducts, the shape of the rib oil ducts (*L. globosa* is an exception), lignification, and the shape of the endosperm in the commissural area. *Lichtensteinia globosa* again appears distinct in having surface protuberances and rib oil ducts unlike those of the other species.

4. Phylogenetic relationships

4.1. Morphological and anatomical phylogenetic analysis

Twenty-five morphological and anatomical characters and their character states are described in Table 1. Maximum parsimony analysis of these data resulted in one tree of 37 steps
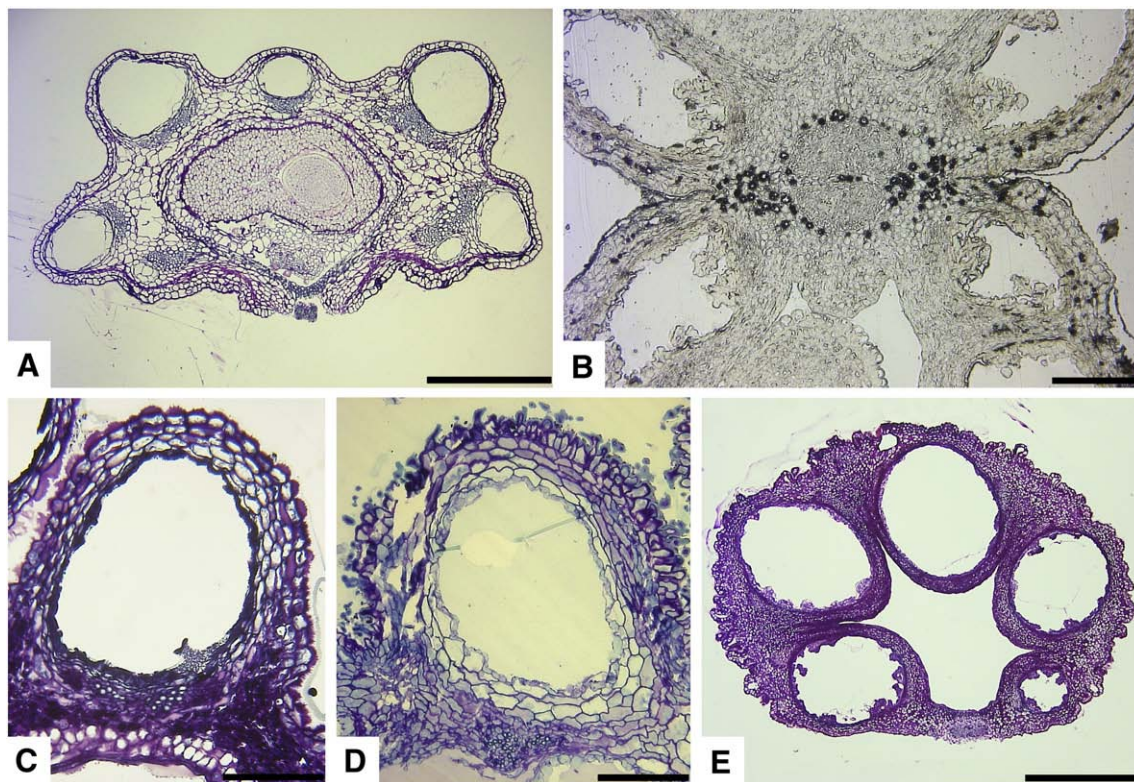
Fig. 9. Transverse sections of portions of the fruits of *Lichtensteinia* species (and Marlothiella) to illustrate some distinctive features. (A) mericarp of *L. lacera* (Boucher 1818) showing the lignified cells that extend beyond the commissure; (B) commissural area of an immature fruit of *L. obscura* (Van Wyk and Tilney 4104) showing the arrangement of crystals in the commissural region; (C) rib oil duct of *L. latifolia* (Rourke 1698) showing the cyclic arrangement of cells surrounding a rib duct; (D) rib oil duct of *Marlothiella gummifera* (Merxmüller and Giess 2344) showing a similar cyclic arrangement of cells; (E) mericarp of *L. globosa* (Magee and Boatwright 33) showing the tuberculate epidermal surface. Scale bars: *A, E*=0.07; *B*=0.02 mm; *C*=0.1 mm; *D*=0.2 mm.

(CI=0.6944 without uninformative characters; RI=0.8197). The resulting tree with synapomorphies mapped along branches is presented in Fig. 10. *Lichtensteinia* is composed of two major groups of species. The thick-rooted, flat-fruited group (i.e., *L. crassijuga*, *L. lacera*, *L. latifolia* and *L. trifida*) is supported by six synapomorphies and a 97% BS value, whereas the *L. interrupta*, *L. globosa* and *L. obscura* group is supported by one synapomorphy and a 55% BS value. Based on this tree, *Lichtensteinia* is not monophyletic, with *Annesorhiza macrocarpa* sister group to one of these clades, and *Marlothiella gummifera* sister group to the other. However, *Lichtensteinia* is supported as monophyletic in trees that are four steps longer than that most parsimonious (no. of MP trees=6; length=41 steps; CI=0.6250 without uninformative characters; RI=0.7541). In these trees, the relationships among *Lichtensteinia* species are the same as those presented in Fig. 10, but relationships

within the *L. interrupta*, *L. globosa* and *L. obscura* clade are unresolved.

4.2. Molecular phylogenetic analysis

Alignment of complete ITS sequences for 19 accessions of *Lichtensteinia* and outgroups resulted in a matrix of 628 positions with none excluded because of alignment ambiguities. Among *Lichtensteinia* accessions, their sequences ranged from 608 to 611 bp. These aligned data included 506 unvarying positions, 65 autapomorphic positions, and 57 parsimony informative positions. Within *Lichtensteinia*, uncorrected pairwise sequence divergence estimates ranged from identity (between two accessions of *L. lacera*) to 4.1% (between *L. obscura* and *L. interrupta*).

Alignment of cpDNA trnQ-trnK sequences for the same 19 accessions resulted in a matrix of 3953 positions, of which 3489
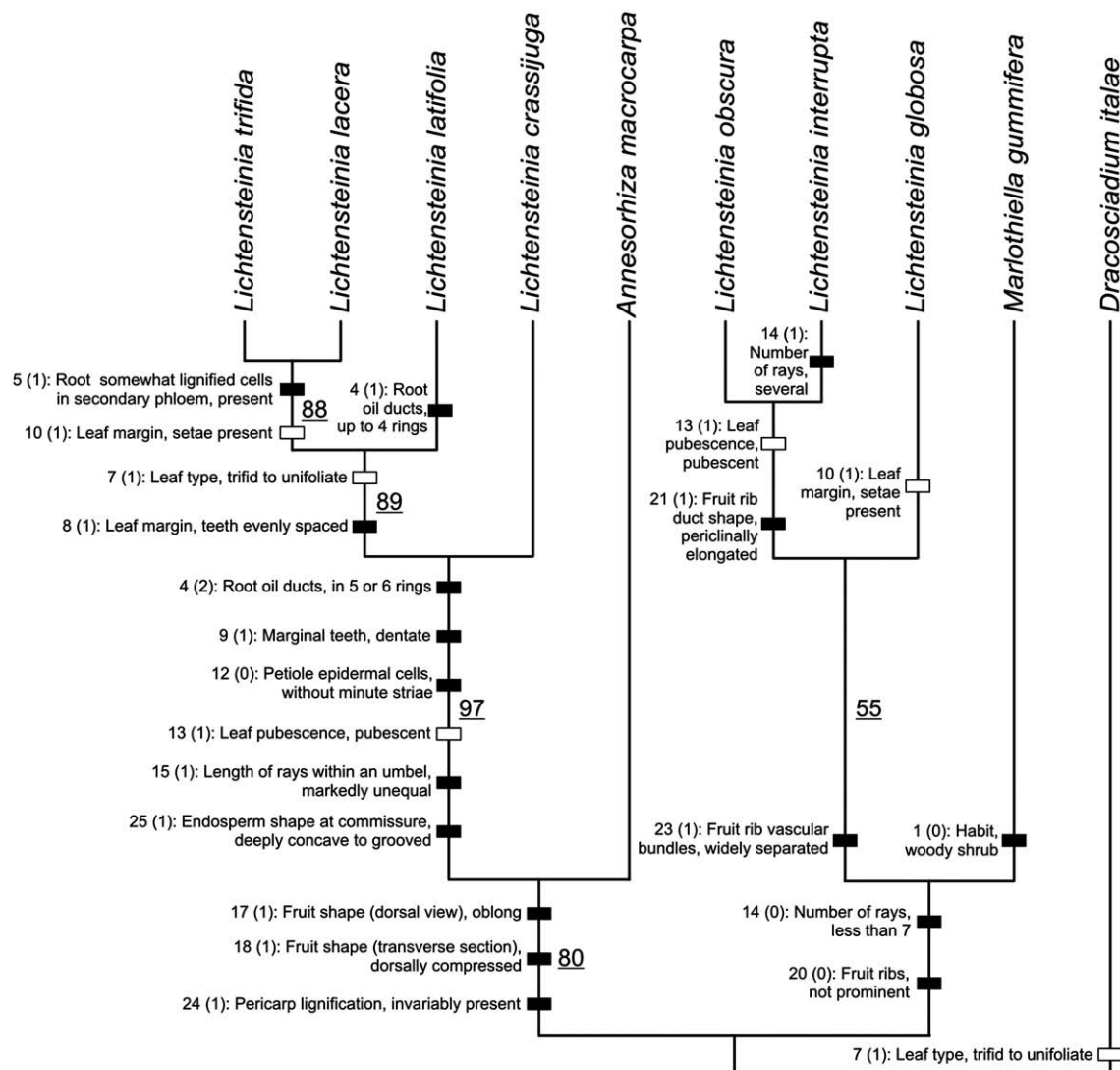
Fig. 10. Minimal length tree inferred from maximum parsimony analysis of 25 morphological and anatomical characters (tree length=37 steps, CI=0.6944 without uninformative characters; RI=0.8197). Synapomorphic characters are mapped along branches, with black and white bars corresponding to non-homoplastic and homoplastic synapomorphic characters, respectively. Numbers to the left of bars correspond to those characters listed in Table 1, with states in parentheses. Characters 2 (root number), 3 (root xylem), 6 (leaf persistence), 11 (leaf lamina), 16 (flower colour), 19 (fruit symmetry), and 22 (concentric rings of cells around rib oil ducts) are not shown because they display ambiguous reconstructions. Underlined numbers are bootstrap estimates for 10,000 replicate analyses (values <50% are not indicated).
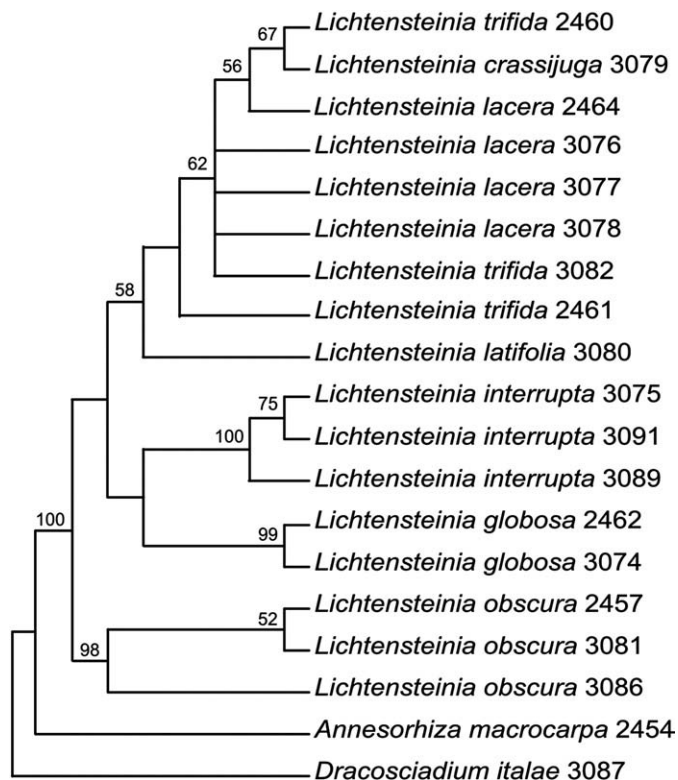
Fig. 11. Strict consensus of four minimal length 171-step trees derived from maximum parsimony analysis of nuclear rDNA ITS sequences (CI=0.7647, without uninformative characters; RI=0.8421). Numbers above branches are bootstrap estimates for 10,000 replicate analyses (values <50% are not indicated).

were unvarying, 320 autapomorphic, and 144 parsimony informative. For 11 accessions, data were missing from the 3'rps16 exon because the endpoints of both forward and reverse primers were located in this highly conserved region. Within *Lichtensteinia*, uncorrected pairwise sequence divergence estimates ranged from 0.1 to 1.7%.

Maximum parsimony analysis of the ITS region resulted in four minimal length, 171-step trees (CI values=0.8586 and 0.7647, with and without uninformative characters; RI=0.8421); the strict consensus of these trees is presented in Fig. 11. While this consensus tree is somewhat resolved, BS values for many branches are weak. In contrast to the previous analysis, the genus *Lichtensteinia* is now supported as monophyletic (100% BS); the genus *Marlothiella*, however, was not included. *Lichtensteinia obscura* comprises a well supported clade (98% BS) that is a sister group to a clade of all other examined species of the genus. *Lichtensteinia interrupta* and *L. globosa* are each well supported as monophyletic, although the sister group relationship between these species is very weakly supported (<50% BS). The clade of *L. interrupta* and *L. globosa* is a sister group to a clade comprising *L. latifolia, L. trifida, L. lacera* and *L. crassijuga*. This group of four species is weakly supported (58% BS) and demonstrates very little resolution of relationships.

Analysis of the cpDNA matrix resulted in a single maximally parsimonious tree of 516 steps (CI values=0.9419 and 0.8333, with and without uninformative characters; RI=0.8770; Fig. 12). Overall, bootstrap support was higher than that of the ITS analysis. Once more, the genus *Lichtensteinia* is supported strongly as monophyletic (100% BS), *Lichtensteinia obscura* is a
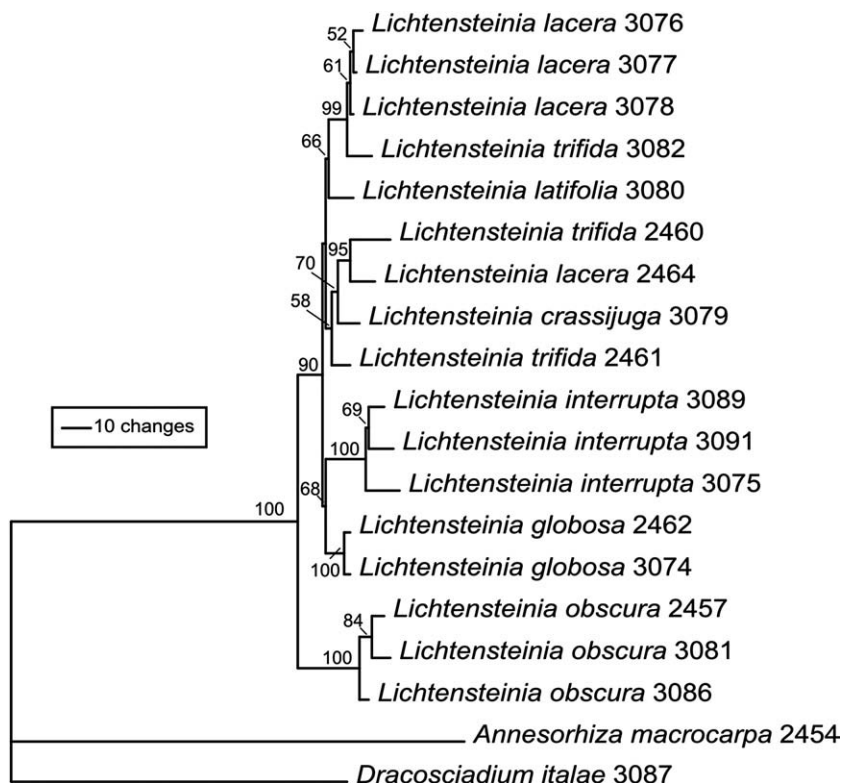

Fig. 12. Single minimal length tree derived from maximum parsimony analysis of cpDNA trnQ-trnK sequences (tree length=516 steps; CI=0.8333, without uninformative characters; RI=0.8770). Numbers above branches are bootstrap estimates for 10,000 replicate analyses (values <50% are not indicated).

Fig. 13. Strict consensus of eight minimal length 691-step trees derived from maximum parsimony analysis of nuclear rDNA ITS and cpDNA trnQ-trnK sequences (CI=0.7972, without uninformative characters; RI=0.8535). An asterisk denotes a different resolution estimated from Bayesian inference (discussed in text). Numbers above branches are bootstrap estimates for 10,000 replicate analyses and posterior probabilities values (expressed as percentages), respectively. Numbers below branches are bootstrap estimates for 10,000 replicate analyses and posterior probabilities values (expressed as percentages), respectively, for the relationships estimated from combined nuclear rDNA ITS, cpDNA trnQ-trnK, and morphological data sets.

sister group to all other examined accessions, and *L. interrupta* and *L. globosa* comprise monophyletic sister groups (68% BS). The clade of *L. interrupta* and *L. globosa* is a sister group to a weakly supported clade (<50% BS) including *L. latifolia*, *L. trifida*, *L. lacera* and *L. crassijuga*. Resolution of relationships within the latter group is improved in comparison to the ITS analysis; *L. lacera* and *L. trifida*, however, are not resolved as monophyletic.

Results of a partition homogeneity test between the ITS and cpDNA data sets reveal that these data partitions are not significantly incongruent ($P=0.14$), therefore they were combined for a simultaneous analysis. Alignment of all sequence data resulted in a matrix of 4581 positions, of which 201 were parsimony informative. Uncorrected pairwise sequence divergence values ranged from 0.1 to 2.0%. Maximum parsimony analysis of these combined data resulted in eight minimal length trees each of 691 steps (CI values=0.9161 and 0.7972, with and without uninformative characters; RI=0.8535). The strict consensus of these trees is presented in Fig. 13. In this analysis, bootstrap support values were generally higher than in any partitioned analyses of molecular data. Once more, the clade of *L. obscura* and the clade of *L. interrupta* plus *L. globosa* are successive sister groups to the *L. latifolia*–*L. trifida*–*L. lacera*–*L. crassijuga* complex. Within the latter, support for three of the five resolved clades is strong (93–98% BS) but as in the partitioned analyses, *L. lacera* and *L. trifida* are each not resolved as monophyletic. Single exemplars of *L. crassijuga* and *L. latifolia* preclude hypotheses on the monophyly of each of

these species. The results of the Bayesian analysis are completely congruent with those inferred by MP, with the exception that *L. trifida* 2461 is resolved as a sister group to all other species of the *L. latifolia*–*L. trifida*–*L. lacera*–*L. crassijuga* complex (albeit this relationship is very weakly supported, with a 52% posterior probability [PP] value).

Results of a partition homogeneity test between the molecular and morphological/anatomical data sets reveal that these data partitions are significantly incongruent ($P=0.01$); nevertheless, they were combined for a total evidence analysis. Maximum parsimony analysis of combined data resulted in four minimal length trees each of 734 steps (CI values=0.8965 and 0.7683, with and without uninformative characters; RI=0.8621). Both MP and Bayesian trees are completely congruent with the results of the cpDNA+ITS analysis, with BS and PP values only slightly lower in some parts of the tree and higher in others (Fig. 13).

5. General discussion

This survey of root, leaf, flower and fruit structure has revealed potentially useful taxonomic characters. In particular, the leaf margins were found to be of diagnostic value in distinguishing among the species. Despite many remaining uncertainties, our field work, herbarium studies and the results of the phylogenetic analyses have resulted in important new insights on *Lichtensteinia* relationships which are discussed below.

Phylogenetic analysis of morphological and anatomical data reveals that *Lichtensteinia* is not monophyletic, because

putative outgroups *Annesorhiza macrocarpa* and *Marlothiella gummifera* were nested within *Lichtensteinia*. *Lichtensteinia* is monophyletic, however, in trees four steps longer than those maximally parsimonious. In contrast, phylogenetic analyses of separate and combined ITS and cpDNA sequences, as well as phylogenetic analyses of combined molecular and morphological/anatomical data, reveal that *Lichtensteinia* comprises a well-supported monophyletic group. However, *Marlothiella* was not included in these molecular or combined analyses. We believe the inclusion of *Annesorhiza* within *Lichtensteinia* is very likely a result of the few morphological and anatomical characters available for analysis. The three characters supporting this relationship occur in several other genera and are likely to represent superficial similarities. The genus *Marlothiella* may be the closest relative of *Lichtensteinia*, based on the shared presence of concentric rings of cells around the large rib oil ducts. This feature is not present in any of the fruits from a large number of genera studied to date (e.g. Liu, 2004; Liu et al., 2003, 2007). Furthermore, *Lichtensteinia* and *Marlothiella* are the only two genera known to us where marked differences in the size of the rib ducts result in the fruits being heteromericarpic (see Fig. 8, E1, F1 and also Fig. 4 in Liu et al., 2007). A relationship between these genera is surprising, as *Marlothiella* is superficially very different — a woody shrublet with succulent leaves. It will be interesting to explore this apparent close relationship when ITS and cpDNA sequence data become available for *Marlothiella*.

The results of the molecular phylogenetic analyses differ from those inferred by analysis of morphological/anatomical data in that *L. obscura* does not fall within the *L. interrupta-L. globosa* clade in the former. Instead, the molecular phylogenies show that *L. obscura* is a sister group to all other species of *Lichtensteinia*. The same relationship is evident as a result of the total evidence analyses. All analyses group *L. latifolia*, *L. trifida*, *L. lacera*, and *L. crassijuga* in one clade. *Lichtensteinia lacera* and *L. trifida*, however, are not monophyletic. The various populations of these two species are morphologically quite distinct (e.g., Fig. 3), with large differences among populations but limited variation within populations. Each species within the complex, however, is easily distinguished morphologically by the size, shape and marginal teeth of its leaves. Further studies of these species are necessary to test if the morphological characters previously used to delimit the species are unreliable and to ascertain what may be causing the discordance in the position of *L. obscura* in the molecular and morphological/anatomical phylogenies.

Lichtensteinia obscura, L. interrupta and *L. globosa* share the following features: generally several relatively thin roots in a cluster frequently having several short lateral roots arising more or less at right angles; leaves that are ±pinnatisect with serrate margins; and fruits that are ovoid, with generally inconspicuous ribs, pericarps that are rarely lignified, and rib vascular bundles that are widely separated. Only the last character, however, is synapomorphic based on the results of phylogenetic analysis of morphological/anatomical characters (Fig. 10). Incomplete specimens of *L. interrupta* and *L. obscura* are easily confused but their leaves are usually quite distinct and

their flower colour is different. The molecular and total evidence analyses did not support *L. obscura, L. interrupta* and *L. globosa* as a monophyletic group and instead showed that *L. globosa* is a sister group to *L. interrupta*, with which it shares several morphological characters (e.g., white flowers, isodiametric mericarps and pinnatisect leaves). The protuberances on the fruit of *L. globosa* are an unusual distinguishing character that is independently found in members of subfamily Saniculoideae. The markedly globose shape of the fruits of this species is also characteristic, as are the relatively small commissural rib oil ducts. The description and nomenclature of the new species will be published elsewhere (Van Wyk and Tilney, in press).

Morphological and anatomical evidence supports the idea of combining *L. kolbeana* and *L. interrupta* into a single species, as anticipated by Burtt (1991). Both have white flowers (flower colour is mostly recorded as yellow in *L. obscura*). Typical *L. kolbeana* is easily distinguished by the larger number of rays, the co-occurrence of leaves and inflorescences and a more eastern distribution, while *L. interrupta* seems to have fewer (up to eight) rays, smaller, proteranthous leaves and a western distribution in the Cape Fynbos region. However, there are numerous intermediate specimens, especially from the southern Cape region. Molecular evidence also supports the treatment of *L. kolbeana* under the synonymy of *L. interrupta* because the specimen of *L. kolbeana* included in the study (Tilney 258; DNA no. 3075) nests within the *L. interrupta* clade in the ITS-derived phylogeny.

Lichtensteinia crassijuga, L. lacera, L. latifolia and *L. trifida* have the following features in common: generally few and relatively thick roots lacking a highly fibrous core; pubescent leaves that are not ±pinnatisect having dentate margins; fruits that are flat, oblong, with fairly conspicuous ribs, pericarps that are lignified to some extent, and rib vascular bundles that are close together or slightly separated. Morphological synapomorphies include root oil ducts in five or six rings, dentate marginal teeth, petiole epidermal cells without minute striae, pubescence on leaves, markedly unequal rays within an umbel, and concave to grooved endosperm at the commissure (Fig. 10).

Despite its seemingly distinctive simple leaves with denticulate margins, the Teewaterskloof population of *Lichtensteinia* (Van Wyk et al. 4210; DNA no. 3082) has exactly the same character states as the other accessions of *L. trifida* and was therefore not included separately in the morphological/anatomical analysis. However, in most other analyses (Figs. 12 and 13), it groups with three of four accessions of *L. lacera* with strong BS support and PP values. It is interesting to note that all of these accessions correspond to the simple leaf forms of *L. lacera* and *L. trifida*. We have observed that populations of *L. trifida* from the Drakenstein Mountains (e.g., at Du Toit's Kloof, Van Wyk and Tilney 4164) have rather small, trifoliate leaves with similarly small marginal teeth. We suggest that the Teewaterskloof population represents a southern form of *L. trifida* with simple leaves, connected to the typical northern trifid forms in the Cedarberg via a trifoliate intermediate, thus giving a similar trend as observed in *L. lacera*.

Of the four species representing the *L. latifolia–L. trifida–L. crassijuga–L. lacera* complex, only *L. latifolia* appears to be

fairly morphologically distinct. In addition to its uniquely dentate leaf margins, the leaves (when simple/unifoliate) have three to five main veins radiating from the base. In all other species, forms with simple/unifoliate leaves have a single and often very prominent midrib. *Lichtensteinia lacera*, *L. trifida* and *L. crassijuga* are very similar morphologically, but are easily distinguished by their leaf margins. It is possible that *L. crassijuga* merely represents an extreme northern form of *L. lacera*, but it is important to note that the marginal teeth of its leaves are diagnostically different, with setaceous teeth completely lacking in the former. The two taxa are also geographically isolated by more than 100 km, with the various forms of *L. lacera* restricted to the Cape Peninsula and the Hottentot's Holland Mountains in the south and *L. crassijuga* endemic to the southern Cedarberg area. Based on the size, shape and number of divisions of the leaf, at least three distinct forms of *L. lacera* can be distinguished (two of which are shown in Fig. 3E,G).

It appears that *L. latifolia*, *L. trifida*, *L.crassijuga* and *L. lacera* may not be good species, with the only differences among them being features of the marginal teeth of their leaves. The molecular phylogenies do not resolve *L. lacera* and *L. trifida* as monophyletic species and the inclusion of only single exemplars of *L. crassijuga* and *L. latifolia* preclude hypotheses on their monophyly. Until further evidence is available suggesting an alternative treatment, we maintain the recognition of four species within this complex.

Acknowledgements

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