

The phylogenetic relationships and evolution of the Canarian laurel forest endemic *Ixanthus viscosus* (Aiton) Griseb. (Gentianaceae): evidence from *matK* and ITS sequences, and floral morphology and anatomy

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Abstract. The phylogenetic relationships of the Canarian laurel forest endemic *Ixanthus viscosus* (Aiton) Griseb. (Gentianaceae) are investigated through a cladistic analysis of sequence variation of parts of the chloroplast gene *matK* and the ITS region of nuclear ribosomal DNA. The floral anatomical and morphological characteristics of *Ixanthus* based on paraffin sections and SEM studies are also discussed. In the molecular analyses *Ixanthus* is part of a clade of mostly temperate Erythraeinae and Chironiinae sensu Gilg, in which it is sister to the mostly Mediterranean annual *Blackstonia*. This relationship is supported by far-reaching similarities in flower morphology and anatomy, and the shared possession of at least some perfoliate leaves. This finding contradicts most former classifications in which *Ixanthus* was placed either in subtribe Gentianinae which never included *Blackstonia*, or in a subtribe of its own which was suggested to connect woody tropical with herbaceous temperate taxa. The phylogenetic relationships found here show that 1) the sister group of *Ixanthus* is distributed in the Mediterranean area, and that 2) its habit as a basally woody suffrutescent herb is best interpreted as an advanced character state.

Key words: Gentianaceae, *Ixanthus*, *Blackstonia*, *matK*, ITS, floral morphology and anatomy, Canary Islands.

Introduction

Oceanic islands have attracted the interest of students of evolutionary process and pattern ever since Darwin's (1859) 'Origin of Species' (e.g. Carlquist 1974, Bramwell 1979, Wagner and Funk 1995, Stuessy and Ono 1998). Problems addressed include the high degree of endemism resulting from radiation processes, the geographical origin of their biotas, and the concentrated occurrence of taxa that diverge strongly from their closest non-island relatives in various morphological characters, including growth habit in plants.

The flora of the Canary Islands harbours some 1270 species of native vascular plants of which ca. 48% have been considered to be endemic (Kunkel 1993). This flora contains elements of Mediterranean, Cape, East African and Neotropical origin (Christ 1885,

Meusel 1965, Bramwell 1976, Francisco-Ortega et al. 1997). In the Canary Islands there are several taxa that differ from the majority of their closest relatives in being woody instead of herbaceous. Such woody species as found in, e.g. *Aeonium* Webb and Berthel., *Echium* L., *Hypericum* L. and *Sonchus* L. (Carlquist 1974), have been interpreted differently. They have either been considered palaeorelicts of a formerly widespread Tertiary laurel forest flora (Meusel 1952, 1965; Lems 1961; Werner 1966; Bramwell 1976; Sunding 1979), or their woodiness has been regarded as being secondary and derived from a herbaceous habit in adaptation to climate and other factors (Schenk 1907; Johnston 1953; Carlquist 1962, 1974, 1984).

The monotypic *Ixanthus* Griseb. (Gentianaceae) is one of 28 endemic genera of the Canary Islands (Kunkel 1993). *Ixanthus viscosus* (Aiton) Griseb. is a perennial, basally woody, and sparsely branched herb which reaches a height of up to 2 m. It occurs only in the Western Canary Islands on Tenerife, Gran Canaria, La Palma, La Gomera and El Hierro (Bramwell and Bramwell 1990, Hohenester and Welss 1993, Kunkel 1993) where it grows in the understorey of laurel forests (*Ixantho-Laurion azoricae* Oberdorfer ex Santos; Rivas-Martínez et al. 1993).

Ever since the revision of the Gentianaceae by Gilg (1895), most authors have regarded *Ixanthus* as being part of subtribe Gentianinae (Gillet 1957, Toyokuni 1963, Smith 1970, Ho and Liu 1990, Yuan and Küpfer 1995). Before that, *Ixanthus* had been included in the Chloreae by Grisebach (1839), and in the roughly synonymous Erythraeae by Bentham (1876). The only cladistic analysis of the phylogenetic relationships of *Ixanthus* was conducted by Meszaros et al. (1996) using morphological characters. In this analysis, *Ixanthus* occupies a position between the neotropical woody *Symbolanthus* G. Don, *Lisianthus* P. Browne and a clade of temperate herbaceous taxa comprising, e.g. *Gentiana* L. and *Centaurium* Hill. Meszaros et al. (1996)

suggested that *Ixanthus* should be interpreted as a relict which may have descended from a neotropical ancestor. In consequence, they interpreted the basal woodiness of this genus as intermediate between the entirely woody and the herbaceous members of the Gentianaceae. *Ixanthus* was also assigned subtribal rank in their study.

To examine the controversial phylogenetic relationships of *Ixanthus*, we sequenced parts of the chloroplast gene *matK* and the ITS region of nuclear ribosomal DNA of various relevant genera of the Gentianaceae. The *matK* gene is relatively conserved (Hilu and Liang 1997) and has been used for phylogenetic analyses at infrafamilial level in, e.g. the Apiaceae (Plunkett et al. 1996), Apocynaceae (Endress et al. 1996), Poaceae (Liang and Hilu, 1996), and Saxifragaceae (Johnson and Soltis 1994). The ITS region has been used extensively for phylogenetic studies at infrageneric level (Baldwin et al. 1995). Macaronesian taxa examined for ITS sequence variation include *Aeonium* (Mes et al. 1996), *Sonchus* and relatives (Kim et al. 1996), and *Argyranthemum* Webb (Francisco-Ortega et al. 1997).

The phylogeny found will be the basis for the interpretation of the ecology, habit and possible geographical origin of *Ixanthus*.

Materials and methods

Taxon sampling: taxa included in our analysis are listed in Table 1. Their classification follows Gilg (1895), Leeuwenberg and Leenhouts (1980), and Endress et al. (1996). Because the phylogenetic relationships of *Ixanthus* within the Gentianaceae were essentially unknown, we used a broad sample of the family for our analysis. Our choice of included taxa is based on 1) results of ongoing molecular phylogenetic studies of the entire family and 2) the classification by Gilg (1895).

Sequence variation of ca. 120 species of the Gentianaceae in the *trnL*-intron (Struwe et al. 1998a, Struwe et al. 1998b) and parts of *matK* (Thiv and Kadereit unpubl. results) have been analyzed phylogenetically. Of the *matK* sequences,

Table 1. Origin of plant material, voucher information, and accession numbers. The classification mostly follows Gilg (1895), Leeuwenberg and Leenhouts (1980) and Endress et al. (1996). *HBM* = plants grown at the Botanic Garden Mainz, *RBGK* = plants grown at the Royal Botanic Gardens Kew, *Herb.* = herbarium material

No.	Taxon	Classification	Source	Collector	Locality	matK 469-849 Genbank number	matK 963-1244 Genbank number	ITS1 Genbank number	ITS2 Genbank number	Voucher
1	<i>Blackstonia acuminata</i> (W. D. J. Koch and Ziz) Domin	Erythraeinae	Silica	Licht	Italy			AJ011468	AJ011478	MIG
2	<i>Blackstonia imperfoliata</i> (L.F. Samp.)	Erythraeinae	HBM 286	Thiv	Italy	AJ010506	AJ011435	AJ011470	AJ011480	MIG
3	<i>Blackstonia perfoliata</i> (L.) Huds. subsp. <i>perfoliata</i>	Erythraeinae	HBM	Thiv	Italy			AJ011469	AJ011479	MIG
4	<i>Canscora pentanthera</i> C. B. Clarke	Erythraeinae	Silica	Mayer 9642	Malaysia	AJ010507	AJ011436			MIG
5	<i>Centaurium maritimum</i> (L.) Fritsch	Erythraeinae	Silica	Licht KC24	Italy	AJ010508	AJ011437	AJ011466	AJ011476	MIG
6	<i>Chelonanthus alatus</i> (Aubl.) Pullé	Helieae	Silica	Thiv	Bolivia	AJ010520	AJ011449			L, PB
7	<i>Chromola baccifera</i> L.	Chrominae	RBGK	Thiv	S-Africa	AJ010509	AJ011438	AJ011464	AJ011474	MIG
8	<i>Cicendia filiformis</i> (L.) Delarbre	Erythraeinae	Herb	Thiv 2156	France	AJ010510	AJ011439	AJ011463	AJ011473	MIG
9	<i>Couroubea tomosa</i> Aubl.	Helieae	Herb.	Jansen-Jacobs 5247	Guyana	AJ010511	AJ011440			U
10	<i>Crowfordia spectiosa</i> Wall.	Gentianaee	Herb.	Keke 1244	Nepal	AJ010512	AJ011441			K
11	<i>Encostena axillare</i> (Lam.) A. Raynal	Erythraeinae	Herb.	Breyne 5540	Tanzania	AJ010513	AJ011442			BR
12	<i>Eustoma grandiflorum</i> (Raf.) Shimmers	Erythraeinae	HBM	Gutsche		AJ010514	AJ011443			MIG
13	<i>Exacum affine</i> Balf. f. ex Regel	Exacinae	HBM	Thiv	Socotra	AJ010515	AJ011444			MIG
14	<i>Fagraea racemosa</i> Jack	Loganiaceae-Potalieae	Herb.	Worthington 12889	Malaysia	AJ010516	AJ011445			NY
15	<i>Gelsaniam sempervirens</i> (L.) J. St Hill	Loganiaceae-Gelsemieae	GenBank			Z70195	Z70195			
16	<i>Gentostoma rupestre</i> J. R. Forst and G. Forst.	Loganiaceae-Loganiaceae	GenBank			Z70194	Z70194			
17	<i>Gentiana cruciata</i> L.	Gentianaee	HBM	Thiv	Germany	AJ010519	AJ011448			MIG
18	<i>Gentianella aspera</i> (Heeg. and Heer) Dostal ex Skalicky, Chrtek and Gill	Gentianaee	Silica	Gutsche 45		AJ010517	AJ011446			MIG
19	<i>Gentianella cerasioides</i> (Kunth) Fabris	Gentianaee	RBGK	Thiv	Ecuador	AJ010518	AJ011447			MIG
20	<i>Ixanthus viscosus</i> (Aiton) Griseb	Gentianaee	HBM	Thiv	Tenenife	AJ010521	AJ011450			MIG
21	<i>Listianthus jefensis</i> A. Robyns	Tachinae	Silica	Luteyn 14796	Panama	AJ010522	AJ011451	AJ011471	AJ011481	MIG
22	<i>Lucilia gratissima</i> (Wall.) Sweet	Rubiaceae-Coposaelleae	GenBank			Z70199	Z70199			NY
23	<i>Macrocarpaea domingensis</i> Urb. and Ekman	Tachinae	Silica	Maas 8395	Dom. Rep.	AJ010523	AJ011452			U

Table 1 (continued)

No.	Taxon	Classification	Source	Collector	Locality	matK 469-849 Genbank number	matK 963-1244 Genbank number	ITS1 Genbank number	ITS2 Genbank number	Voucher
24	<i>Neurotheca laevifolia</i> (Spruce ex Proge) Baillon	Erythraeaceae	Herb.	Van deelen Berg 4957	Senegal	AJ010524	AJ011453			BR
25	<i>Orphium frutescens</i> (L.) E. Mey	Chromolaenaceae	RBGK	Thiv	S-Africa	AJ010525	AJ011454	AJ011465	AJ011475	MJG
26	<i>Sabaia angulata</i> (L.) Push	Erythraeaceae	Herb.	Lammers 4860	USA	AJ010526	AJ011455	AJ011467	AJ011477	NY
27	<i>Schinzeria tetragona</i> (Schinz) Gilg	Erythraeaceae	Herb.	Malasse 13852	Zaire	AJ010527	AJ011456			BR
28	<i>Strychnos nux-vomica</i> L.	Loganiaceae	GenBank			Z70193	Z70193			
29	<i>Sweria peruviana</i> L.	Strychnaceae	HB Munich		Germany	AJ010528	AJ011457			MJG
30	<i>Symbolanthus calygonus</i> (R and P) Griseb	Gentianaceae	Silica	Guische 56 Thiv	Bolivia	AJ010529	AJ011458			LPB
31	<i>Symphylitphyton caprifolioides</i> Gilg	Helicaceae	Herb.	Ratter 6742	Brasil	AJ010530	AJ011459	AJ011462	AJ011472	F
32	<i>Tachia guianensis</i> Aubl.	Tachinae	Herb.	Rova 1963	Brasil	AJ011433	AJ011461			S
33	<i>Tachadenus carinatus</i> (Desr.) Griseb.	Tachinae	Herb.	Lowry 4551	Madagascar	AJ011434	AJ011460			BR
34	<i>Thevetia peruviana</i> (Pers.) K Schum.	Apocynaceae	GenBank			Z70188	Z70188			

27 were selected for the present study (Table 1). These represent all major clades of the family as found in the highly congruent phylogenies obtained from the analyses of *trnL*-intron and *matK* sequence variation. At the same time most tribes and subtribes as defined by Gilg (1895) were considered for the *matK* analysis (Table 1). In order to test the relationship of *Ixanthus* to subtribe Gentianinae as suggested by Gilg (1895) and other authors, the two major lineages found by Yuan and K pfer (1995) in Gentianinae were represented by *Gentiana* and *Crawfordia* Wall. (*Gentiana*-lineage) and *Gentianella* Moench and *Swertia* L. (*Gentianella*-lineage). The woody neotropical *Lisianthus*, *Macrocarpaea* Gilg, *Symbolanthus* G. Don and *Tachia* Aubl., of which *Lisianthus* and *Symbolanthus* have been suggested to occupy a position immediately below *Ixanthus* by Meszaros et al. (1996), were also included in the study. Furthermore, mostly herbaceous taxa belonging to the palaeotropical Exacinae (*Exacum* L.), Tachiinae (*Tachiadenus* Griseb.), the neotropical Helieae (*Coutoubea* Aubl., *Irlbachia* Mart., *Symphyllphyton* Gilg), the South African Chironiinae (*Chironia* L., *Orphium* E. Mey.), and subtribe Erythraeinae (*Blackstonia* Huds., *Centaurium* and *Cicendia* Adans. from the Mediterranean area and America, *Sabatia* Adans. from North America, and *Canscora* Lam., *Enicostema* Blume, *Neurotheca* Salisb. ex Benth. and *Schinziella* Gilg from Africa and Asia) were analyzed. Accepting the redundancy of the tribes Rusbyanthae (Weaver 1974) and Leiphaimeae (Raynal 1967, Maas and Ruyters 1986), only the mycotrophic genera *Voyria* Aubl. and *Voyriella* Miq. of tribe Voyrieae were not considered in this study. The comparison of ITS1 sequences of *Voyria*, *Voyriella* and *Ixanthus* suggests, however, that these three genera are not closely related to each other (Thiv, unpubl. results). Following Struwe et al. (1994), Bremer (1996), and the results from the *trnL*-intron and *matK* analyses, tribe Potalieae of the Loganiaceae sensu Leeuwenberg and Leenhouts (1980) is part of the Gentianaceae. Accordingly, *Fagraea* Thunb. also was included in our analysis as a representative of this tribe. *Saccifolium* Maguire and Pires as another new element of the family (Struwe et al. 1998a, 1998b) remains unconsidered. Thus, our taxon sample represents almost all tribes and subtribes of the Gentianaceae as described by Gilg (1895). In

accordance with the position of the Gentianaceae in the Gentianales (Chase et al. 1993, Olmstead et al. 1993, Struwe et al. 1994, Bremer 1996), members of the Loganiaceae, Apocynaceae and Rubiaceae were chosen as outgroups for the *matK* analysis.

Following the results of the *matK* analysis and *trnL*-intron data (Struwe et al. 1998b), the clade containing *Blackstonia*, *Centaurium*, *Chironia*, *Cicendia*, *Ixanthus*, *Orphium* and *Sabatia* was analyzed for ITS sequence variation to obtain additional and possibly more detailed evidence for the phylogenetic relationships of *Ixanthus*. ITS sequences were also used to estimate the possible age of the split between *Blackstonia* and *Ixanthus* by comparison with published substitution rates from other taxa. The ITS analysis was rooted using *Symphyllphyton*. ITS sequences of representatives of the other major clades found in the *matK* analysis are too divergent for unambiguous alignment.

DNA extraction: DNA was extracted from ca. 10 × 10 mm silica dried, fresh or herbarium leaf material according to Doyle and Doyle (1987) followed by washing, cleaning, and elution steps using QIA G20 Genomic Kit (Qiagen), or following the protocol of Struwe et al. (1998b).

Amplification: double-stranded products of the chloroplast gene *matK* and the ITS region of nuclear ribosomal DNA were amplified using standard PCR. Because of the use of herbarium material for some of the taxa only two relatively short regions of the *matK* gene could be amplified. Together they comprise ca. 700 nucleotides (Fig. 1). Internal primers for *matK* were designed after searching for conserved regions by using the initial primer *trnKII* (Steele and Vilgalys 1994). Primers used were *matK* part 1: 1198F = 5'-CTGTGTTA-GATATACGAATACC-3' and 1581R = 5'-CTTGATACCTAACATAATGCAT-3', *matK* part 2: 1729F = 5'-AAGGGTCTATATAAAGCAATT-3' and 2053R = 5'-TTAGCRCAAGAYAGTCGAAGTA-3'; ITS1: ITS A = 5'-GGAAGGAGAAGTCGTAA-CAAGG-3' (see Blattner 1996), ITS C = 5'-GC AATTCACCAAGTATCGC-3'; ITS2: ITS D = 5'-CTCTCGGCAACGGATATCTCG-3', ITS B = 5'-CTTTTCCTCCGCTTATGATATG-3'.

Cleaning of PCR products: amplified DNAs were checked on 1.4 or 1.2% agarose gels. Only PCRs showing unambiguous bands were purified

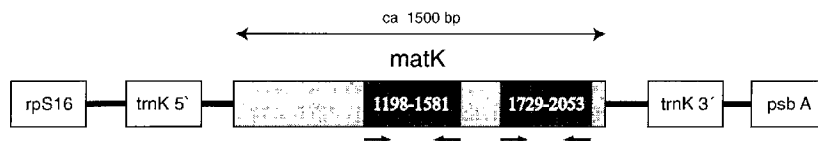


Fig. 1. Amplified region of the *matK* gene. Arrows indicate position of PCR primers used. Boxed areas represent coding regions and connecting lines represent spacer regions

using the QIAquick PCR Purification Kit (Qiagen, Germany) according to the manufacturer's protocol, or following the protocol of Struwe et al. (1998b).

Sequencing reaction: cycle sequencing reactions in both directions were performed using purified double-stranded DNA, thermosequenase Kit (Amersham) and the primers used in the amplification PCR as sequencing primers. Samples were analyzed with automated sequencing systems (P-E Applied Biosystems ABI 373, 377).

Sequence alignment: the relatively conserved *matK* sequences were aligned manually, and the more variable ITS sequences were aligned using Sequencher 3.0 (GeneCodes) followed by manual improvement.

Data analysis: Fitch parsimony analyses were conducted using PAUP (version 3.1.1, Swofford 1993). For the *matK* data set a heuristic search and bootstrap analysis (Felsenstein 1985) (100 replicates) were performed with the ACCTRAN, MULPARS, TBR, and random addition (100 replicates) options. For the ITS data set parsimony and bootstrap analyses (100 replicates) were conducted using the branch-and-bound algorithm. In both data sets gaps were coded as missing data. Transversion parsimony searches were carried out for both data sets. In these, a transition/transversion ratio of 2:1 was used on the basis of the approximate ratio of transitions and transversion in the strict consensus trees of the unweighted analyses. The trees obtained in the unweighted analysis were assessed with Bremer ("decay") indices (Bremer 1988) using PAUP. Consistency index and retention index were also computed. Jukes-Cantor distances were estimated using PHYLIP 3.05 (Felsenstein 1993). For the ITS data set, relative rate tests between *Symphyllphyton* and *Ixanthus/Sabatia*, *Cicendia* and *Ixanthus*/all species of *Blackstonia*, and *Sabatia* and *Ixanthus/Blackstonia imperfoliata* were calculated accord-

ing to Sarich and Wilson (1973). Using Jukes-Cantor distances the possible age of *Ixanthus* was calculated following Li and Graur (1991).

Morphological and cytological data: morphological and cytological characters were compiled from herbarium material (MJG), plants cultivated at the Botanic Garden Mainz, and the literature (Nilsson 1967, Zeltner 1970, Punt and Nienhuis 1976, Meszaros et al. 1996). Chromosome counts were made using the method described in Sauer and Thiv (1995). Microscopic slides of cross-sections of paraffin-embedded flowers of *Blackstonia perfoliata* (P. Endress 7166) and *Ixanthus viscosus* (P. Endress 6739) were studied. The slides were provided by P. Endress and are deposited at Zürich University. SEM studies were made of floral buds and flowers of *Blackstonia perfoliata* (P. Endress 7166) and *Ixanthus viscosus* (Struwe 1079, NY).

Results

matK. The aligned sequences of the two combined parts of *matK* have a length of 684 base pairs (bp). Sequence variation is 59% including and 49% excluding outgroup taxa. Of these 684 bp, 214 (31%) are potentially informative. The unweighted search resulted in 108 most parsimonious cladograms (L (length) = 741, CI (consistency index) = 0.683, RI (retention index) = 0.739). The strict consensus tree (Fig. 2) shows three major lineages within the Gentianaceae. The first (A) consists of the monophyletic subtribe Exacinae. The second (B) contains woody neotropical taxa belonging to Gilg's Helieae and Tachiinae (D), members of subtribe Erythraeinae of mostly African distribution together with *Fagraea* and *Lisianthus* (E), and the monophyletic subtribe

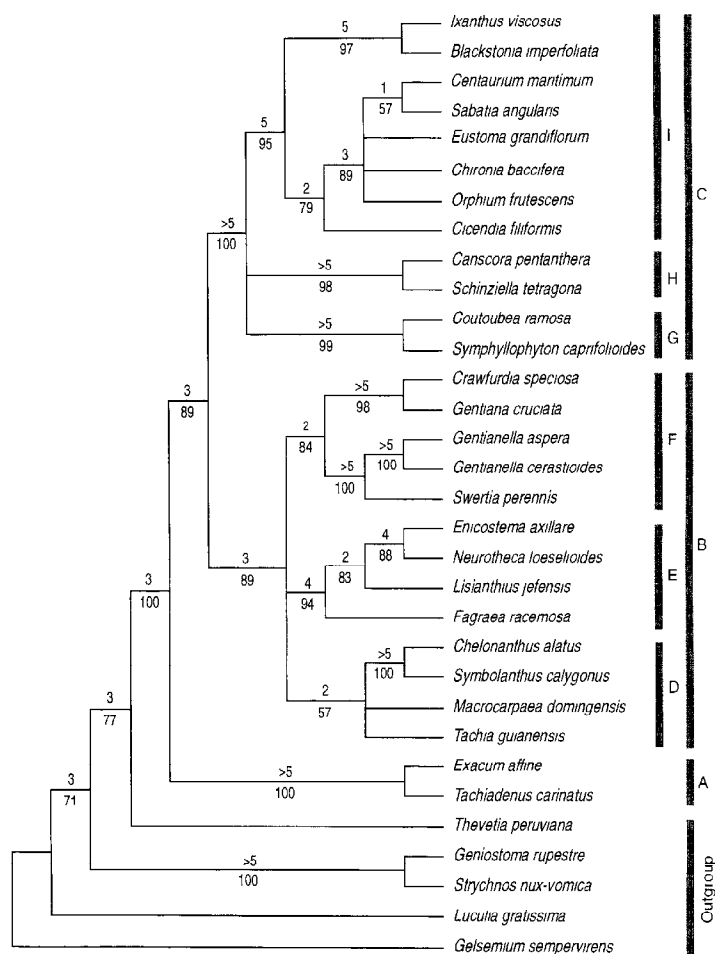


Fig. 2. Strict consensus tree of 108 most parsimonious trees (741 steps; CI = 0.683 (with autapomorphies); RI = 0.739) from the *matK* analysis. Bootstrap values (below branches) and decay indices (above branches) are indicated for each node. Lines to the right define clades

Gentianinae (F). Finally, the third major group (C) includes herbaceous genera of tribe Heliceae (G), *Canscora* and *Schinzia* (H), and taxa of temperate Erythraeinae and Chironiinae as well as the North to Central American *Eustoma* (I). *Ixanthus* is part of this last clade (I), and its sistergroup relationship to *Blackstonia* is well-supported with a bootstrap value of 93% and a decay index of 5. An identical topology was obtained in the transversion parsimony analysis (not shown).

ITS. The length of ITS1 in the taxa surveyed ranges from 230 (*Symphyllphyton*

caprifolioides) to 236 bp (*Ixanthus viscosus*). Sequence variation is 47% including and 39% excluding the outgroup. Of 243 bp, 52 (21%) are potentially informative. Length of ITS2 varies from 230 (*Centaurium maritimum* and *Sabatia angularis*) to 235 bp (*Cicendia filiformis*). Sequence variation is 50% including and 40% excluding outgroup taxa. Of 238 bp, 46 (19%) are potentially informative. In *Ixanthus viscosus* three polymorphisms in ITS2 were found. The ITS unweighted analysis resulted in three most parsimonious cladograms (L = 335, CI = 0.839, RI = 0.752)

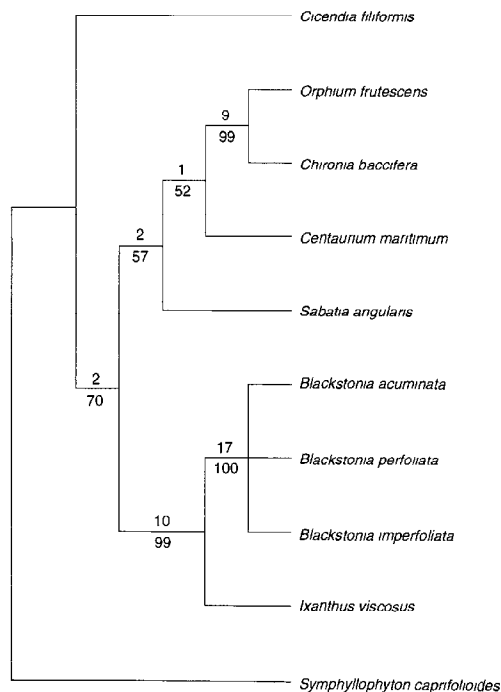


Fig. 3. Strict consensus tree of three most parsimonious trees (335 steps; CI=0.839 (with autapomorphies); RI=0.752) from the ITS analysis. Bootstrap values (below branches) and decay indices (above branches) are indicated for each node

that differ only in the relationships among the three species of *Blackstonia*. The strict consensus tree of these three cladograms is shown in Fig. 3. The ITS results confirm the sister group relationship of *Ixanthus* and a monophyletic *Blackstonia*. A clade containing *Centaureum*, *Sabatia* and Chironiinae (*Chironia*, *Orphium*) is sister to *Ixanthus* and *Blackstonia*. *Cicendia* is sister to these two clades. The clades of *Ixanthus* and *Blackstonia* as well as the clade containing the three species of *Blackstonia* are well-supported by bootstrap values of 99% and 100%, respectively, and decay indices of 10 and 17. Relationships among species of *Blackstonia* could not be resolved due to lack of informa-

tive characters. The cladograms obtained from transversion parsimony have identical topologies (not shown). The deletion of problematical regions of alignment (particularly in relation to the outgroup: bps 40–41, 81–88, 258–261, 277–297) or the coding of gaps as separate characters did not result in any change of phylogenetic topology.

Results of the relative rate test show no significant differences between any of the lineages tested. In consequence, a molecular clock hypothesis can not be rejected.

Morphology (Table 2). The floral anatomy of *Ixanthus viscosus* has not been studied previously as far as we know. *Blackstonia* was investigated by Lindsey (1940; as *Chlora*). The present study primarily focuses on the anatomy of the pedicel and gynoeceum as cross-sections of the upper parts of the calyx, corollas, and anthers were not available.

Ixanthus and *Blackstonia* have typical Gentianaceae flowers in that they are hypogynous, sympetalous, with isomerous stamens, and with a bicarpellate ovary. *Ixanthus* commonly has 5-merous flowers but 4-merous flowers can be found in the terminal position in the panicle (L. Struwe and V. Albert, pers. obs.; Fig. 4A, B). The flowers of *Blackstonia*, on the other hand, are unusual in the Gentianaceae by having 8–12 corolla lobes and stamens (being 8-merous in the investigated specimen of *Blackstonia perfoliata*; Fig. 4A). The following description is based on microscopic slides with cross-sections of partial flowers as well as SEMs of very young floral buds and mature ovaries of *Blackstonia perfoliata* and *Ixanthus viscosus*.

In the floral primordia of *Blackstonia* and *Ixanthus*, the calyx lobes develop and mature much earlier than the rest of the flower and act as a protective cover over the inner, still developing, primordia. Colleters, small finger-shaped multicellular glands in the axils of the leaves and at the inner bases of the calyces, are present in both taxa (Figs. 4A, 5A). Corolla and stamen primordia develop nearly simultaneously in two whorls (Figs. 4A-B, 5A). Each

Table 2. Floral anatomical characteristics of *Ixanthus*, *Blackstonia*, *Gentiana*, *Lisianthus*, and *Symbolanthus*. Data presented in this paper and from Gopal Krishna and Puri (1962), van Heusden (1986), Lindsey (1940), Lindsey (1940), Struwe et al. (1997), Weaver (1972), and Woodson (1938)

	<i>Ixanthus</i>	<i>Blackstonia</i>	<i>Gentiana</i>	<i>Lisianthus</i>	<i>Symbolanthus</i>
stigma shape	bilamellate, two lobes, strongly papillose	bilamellate, four-lobed at apex, two lobes basally, strongly papillose	bilamellate, two lobes, papillose	capitate (rarely slightly bilamellate)	broadly bilamellate, two lobes, strongly papillose
epidermis on ovary and lower part of style	papillose and glandular(?), the cuticle thick and obscurely spiny	papillose and glandular(?), the cuticle thick and obscurely spiny	not investigated	not investigated	not investigated
style shape in cross-section	filiform, with deep sutures	filiform, with deep sutures	flattened, sutures present	filiform, sutures present	filiform, flattened when older, sutures present
postgenital fusion of carpels	present throughout the ovary except at the very base	present throughout the ovary except at the very base	present throughout the ovary except at the very base	present throughout the ovary except at the very base	present throughout the ovary except at the very base
placentation in upper part of ovary	placenta "falsely" axile, due to protrusion of parietal placentas	placenta parietal	placenta parietal, not protruding	placenta "falsely" axile, due to protrusion of parietal placentas	placenta "falsely" axile, due to protrusion of parietal placentas
placentation in basal part of ovary	placenta parietal, protruding and inrolled	placenta parietal, protruding and inrolled	placenta parietal, not protruding	placenta parietal, protruding and inrolled	placenta parietal, protruding and inrolled
ovary position	sessile	sessile	usually stipitate	sessile	sessile
base of ovary	glandular, no prominent disk	glandular, no prominent disk	with prominent glandular disk	with prominent glandular disk	with prominent glandular disk
stamen-corolla tube	late development	late development	late development	late development	late development
stele	circular, not stellate	circular, not stellate	circular, not stellate	circular, not stellate	stellate

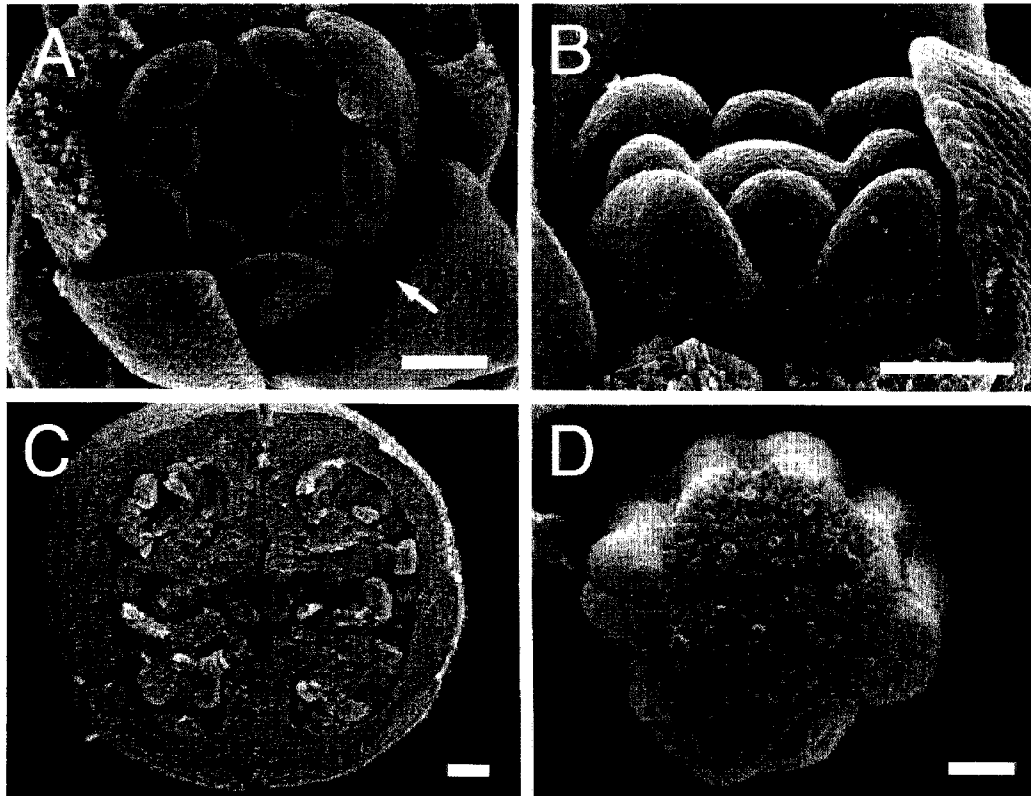


Fig. 4. Flower development and morphology of *Ixanthus viscous* (Struwe 1079). **A** Five-merous flower primordium with partially removed calyx lobes, colleters at the base of the calyx lobes, five petal primordia, five staminal primordia, and an undifferentiated gynoecial primordium in the centre. Arrow indicates colleters. Scalebar = 100 μ m. **B** Four-merous flower primordium showing the same features as in A. Scalebar = 100 μ m. **C** Cross-section of mature ovary with parietal, protruding placentae, and many ovules. Note the incomplete fusion of the two carpels. Scalebar = 200 μ m. **D** Papillose stigma of mature flower shown from above. Scalebar = 100 μ m

primordium is distinct and the fused staminal-corolline tube forms very late in the development of the flower. No signs of early sympetaly could be seen (cf. Erbar 1991). The corolla lobes are contorted. The ovary develops soon after the formation of the corolla lobes and stamens, initially as two separate, superior carpels that fuse postgenitally (Fig. 5A). The development of the corolla, stamen, and ovary primordia is identical in these two taxa apart from the number of corolla lobes and anthers.

Ixanthus and *Blackstonia* both have a circular stele in the receptacle with no tendency towards a stellate arrangement below the level where the calycine traces depart. The calyx is supplied by as many traces as lobes and the traces depart from the stele at the same level. Each calyx trace soon divides into three. After the separation of the calyx the rest of the flower is shortly stalked in *Ixanthus* and the parenchymatous central tissue has specialized, pink-staining (with safranin and astrablue), thick-walled, circular cells that appear to

represent sclereids. Neither of these two traits, however, was seen in *Blackstonia*. The calyx lobes are imbricate. The epidermis on the inside of the calyx tube is glandular and papillose at the base and is developed into individual colleters slightly higher up.

The corolla and stamens are initially supplied by as many vascular bundles as there are lobes/organs. The staminal traces divide later (i.e. more distally) from the stele than the corolline traces.

The ovary is postgenitally fused except for the most basal part in *Blackstonia* and *Ixanthus*. In *Blackstonia* the gynoecium is unilocular throughout the length of the ovary (also below the level of postgenital fusion) with parietal placentae that are inrolled nearly to form a semicircle (Fig. 5C). A close-up of the zone with postgenital fusion in a mature ovary of *Blackstonia* is shown in Fig. 5B. The same ovary morphology is present in *Ixanthus* (Fig. 4C), except for the uppermost part of the

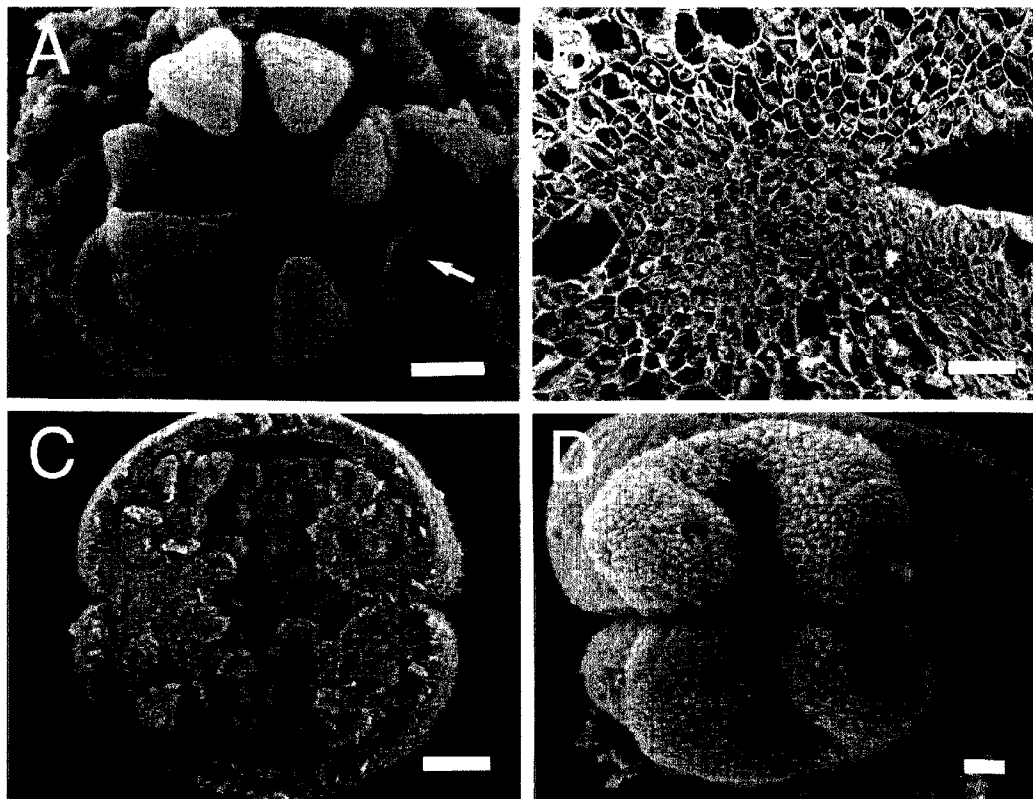


Fig. 5. Flower development and morphology of *Blackstonia perfoliata* (P. Endress 7166) **A** Eight-merous developing flower bud with removed calyx and corolla lobes (except one corolla lobe), colleters at the base of the removed calyx lobes, eight stamens, and developing bicarpellate ovary in the centre. Note the lack of fusion between the two carpels. Arrow indicates colleters. Scalebar = 100 μ m. **B** Close-up of postgenitally fused area of a mature ovary wall. Scalebar = 20 μ m. **C** Cross-section of mature ovary with parietal, protruding placentae, and many ovules. Note the incomplete fusion of the two carpels. Scalebar = 500 μ m. **D** Papillose, twice divided, stigma of mature flower (from above). Scalebar = 100 μ m

Table 3. Morphological, karyological, chemical and ecological comparison between *Ixanthus* and *Blackstonia*. For sources of data see text

	<i>Ixanthus</i>	<i>Blackstonia</i>
life form	perennial	annual
habit	suffrutescent herb, <2 m high	rosette herbs, <0.5 m high
leaves	bracts perfoliate, cauline leaves free	bracts and cauline leaves +/- perfoliate
leaf colour	green	glaucous
inflorescence	paniculate dichasia	dichasia
corolla colour	yellow	yellow
pollen	tricolporate, striate	tricolporate, striate
chromosome number (own counts and Zeltner 1970)	2n ≈ 62	2n = 20 and 40
oxygenation of xanthone position (Meszaros and al. 1996)		
C2	present	absent
C4	absent	absent
C5	absent	absent
C6	present	absent
C7	absent	present
C8	present	present
habitat (Rivas-Martínez and al. 1993, Zeltner 1970)	laurel forests	open, often secondary vegetation

ovary where the placentae meet in the centre creating a septum, i.e. a partly bilocular ovary.

Each carpel in *Blackstonia* and *Ixanthus* receives three vascular bundles. There are six main vascular bundles leading to the ovary, one dorsal and two ventral bundles per carpel (Gopal Krishna and Puri 1962: Fig. 95). Additional smaller bundles are also present in the intruding parts of the parietal placentae, especially in *Ixanthus*, and in the ovary wall. The surface of the ovary is probably glandular, i.e. with thick-celled, dark-staining, papillate epidermis cells, in both *Blackstonia* and *Ixanthus*. The upper part of the ovary as well as the style continues to have a very thick, probably glandular, epidermis and also has a thick cuticle with a serrate surface pattern in cross-section. The sutures between the two carpels are deeply sunk, giving rise to one distinct furrow on each side of the pistil and ovary (Figs. 4C, 5C).

The terminal stigma of each taxon has a densely papillose surface (Figs. 4D, 5D).

Blackstonia has a rather deeply bilobed stigma with each lobe divided into two higher up, i.e., with four stigmatic lobes in total (Fig. 5D). *Ixanthus* has a shallowly bilobed stigma without further division of the stigmatic parts (Fig. 1D). The stigmatic areas on the stigma lobes are pointing outwards-upwards in *Blackstonia*, but inwards-upwards in *Ixanthus*.

The comparison of further morphological characters, chromosome number, ecology and xanthone chemistry of *Ixanthus* and *Blackstonia* is shown in Table 3.

Discussion

The phylogenetic relationships of Ixanthus

The results of the separate analyses of *matK* and ITS sequence variation clearly indicate that *Ixanthus* forms neither part of subtribe Gentianinae nor of the neotropical woody

Gentianaceae, but rather belongs to a clade of mostly temperate taxa including *Blackstonia*, *Centaurium*, *Cicendia*, *Eustoma*, *Sabatia*, and *Chironiinae* (Figs. 2 and 3). In this clade, it is well-supported as sister group to *Blackstonia*. These findings are consistent with the results obtained from the analysis of *trnL*-intron data (Struwe et al. 1998b).

A close relationship of *Ixanthus* to *Blackstonia* had already been suggested in the past by Grisebach (1839) who had placed *Ixanthus* in tribe Chloreae together with *Chlora* Adans. (= *Blackstonia*), *Sabatia* and *Schultesia* Mart. A similar classification was proposed by Bentham (1876) who included *Ixanthus* in subtribe Erythraeae (= Erythraeinae) comprising *Canscora*, *Chlora*, *Cicendia*, *Coutoubea*, *Deianira* Cham. and Schltldl., *Erythraea* L., *Neurotheca*, *Sabatia*, *Schultesia*, and *Zygostigma* Griseb. Also Christ (1885) and Schenk (1907) had emphasized the similarity of *Ixanthus* and *Blackstonia* in vegetative characters. Gilg's (1895) inclusion of *Ixanthus* in subtribe Gentianinae was based on the possession of unilocular ovaries and large single pollen grains with an exine structure that was misinterpreted as scabrate rather than striate (Nilsson 1967, Punt and Nienhuis 1976, Nilsson, pers. com.). Within the subtribe, Gilg (1895) grouped *Ixanthus* with *Crawfordia*, *Gentiana* and *Jaeschkea* Kurz, which are characterized by corollas without nectaries and non-sessile stigmata. Although Gilg's (1895) treatment received much criticism (e.g. Köhler 1905), most authors (Gillet 1957, Toyokuni 1963, Smith 1970, Ho and Liu 1990, Yuan and Küpfer 1995) followed Gilg (1895) and placed *Ixanthus* in subtribe Gentianinae. Recently, Meszaros et al. (1996) recognized the incorrect position of *Ixanthus* within this subtribe. They hypothesized that *Ixanthus* as a palaeorelict connects phylogenetically more basal woody tropical taxa with derived herbaceous temperate Gentianaceae. In their analysis, the characters excluding *Ixanthus* from the herbaceous temperate clades of the family are the

oxygenation patterns of xanthenes in two positions.

Blackstonia, the sister genus of *Ixanthus*, comprises four annual species (Zeltner 1970). These are *Blackstonia acuminata* (W. D. J. Koch and Ziz) Domin (with two subspecies), *B. grandiflora* (Viv.) Pau, *B. imperfoliata* (L.f.) Samp., and *B. perfoliata* (L.) Huds. (also with two subspecies). All four species have a diploid chromosome number of $2n = 20$, but *B. acuminata* and *B. perfoliata* also contain tetraploid subspecies with $2n = 40$. Whereas *B. grandiflora* and *B. imperfoliata* are West to Central Mediterranean in distribution, the diploid subspecies of *B. acuminata* and *B. perfoliata* are more widespread in the Mediterranean basin. The tetraploid subspecies of the latter two species also are found in W and C Europe (Zeltner 1970, Meusel et al. 1978). Although *B. grandiflora*, supposedly the most primitive species of the genus (Zeltner 1970), was not included in our analysis, the morphological homogeneity of *Blackstonia* leads us to believe that this genus is monophyletic rather than paraphyletic in relation to *Ixanthus*.

The floral anatomy and morphology of *Ixanthus* is more similar to *Blackstonia* (subtribe Erythraeinae) than to either *Gentiana* (Gentianinae), *Lisianthus* (Tachiinae), or *Symbolanthus* (tribe Helieae; see Table 1 for an overview). Distinct differences from *Gentiana* are the presence of a long style as opposed to a nearly sessile stigma and a well-developed, bilobed, protruding placenta instead of ovules distributed on a thin placenta covering large areas on the inner ovary wall (Lindsey 1940, Gopal Krishna and Puri 1962). Both *Symbolanthus* and *Lisianthus* have parietal, protruding, inrolled placentae that are also characteristic of *Ixanthus* (Van Heusden 1986). However, the corollas of *Lisianthus* have prominent fiber bundles, a feature that is absent from *Ixanthus* (Woodson 1938, Lindsey 1940). The flowers of *Symbolanthus* are also very different from *Ixanthus* in having corollas with a corona at the base of the filaments, a stellate stele in the receptacle,

and a prominent disk at the base of the ovary (Van Heusden 1986, Struwe et al. 1997).

The only major difference between *Blackstonia* and *Ixanthus*, apart from the number of corolla lobes and stamens, is the stigma morphology. *Blackstonia* has a twice bilobed stigma with the stigmatic surface facing outwards-upwards as opposed to the shallowly bilobed stigma with stigmatic surfaces towards the inside of the lobes in *Ixanthus*. The difference in floral merosity is interesting since this characteristic seems to have evolved three times independently in the Gentianaceae. Except for *Blackstonia*, eight or more corolla lobes are present in *Sabatia* (subtribe Erythraeinae) and in the two closely related genera *Anthocleista* and *Potalia* (tribe Potalieae, former Loganiaceae; Struwe et al. 1994, Struwe and Albert 1997, Leeuwenberg and Leenhouts 1980).

Beyond floral anatomy and morphology, the comparison of *Ixanthus* and *Blackstonia* (Table 3) shows dissimilarities between the two taxa in life form and habit, leaf colour, inflorescence morphology, xanthone chemistry, chromosome number and ecology. This may illustrate Carlquist's (1974) conclusion that the characters of insular taxa often diverge strongly from their continental relatives. *Ixanthus* and *Blackstonia* are similar to each other in having at least some perfoliate leaves, yellow flowers, and tricolporate-striate pollen grains. None of these characters is unique to the *Ixanthus/Blackstonia* clade. Whereas yellow flower colour and tricolporate-striate pollen grains are widespread in the family and among the close relatives of *Ixanthus/Blackstonia*, perfoliate leaves are less common and are found only in *Canscora* and *Symphyllophyton* of those genera included in our clade C (Fig. 2). In consequence, the presence of perfoliate leaves could be interpreted as a homoplastic synapomorphy further supporting the sister group relationship between *Ixanthus* and *Blackstonia* as revealed by the analysis of molecular characters. The chromosome number of *Ixanthus viscosus* ($2n=62$) could be

interpreted as a hexaploid and at the same time dysploid derivative of a diploid common ancestor with $2n=20$ chromosomes as found in *Blackstonia* (Zeltner 1970). The three polymorphisms found in *Ixanthus viscosus* do not allow an interpretation of the evolution of chromosome number (c.g. auto- vs. allopolyploidy) in *Ixanthus*.

Geographical relationships, growth form, age and habitat of Ixanthus

Blackstonia has a clear centre of diversity in the Mediterranean area (Zeltner 1970, Greuter et al. 1986) and has been suggested to have originated and radiated in Morocco (Zeltner 1970). A sister group relationship of endemic genera of the Canary Islands to Mediterranean taxa recently was also found for, e.g. *Agyranthemum* (Francisco-Ortega et al. 1997; see also for further literature) and *Isoplexis* (Carvalho and Culham in press). Although 1) *Eustoma* and *Sabatia* as part of our clade C are of North to Central American distribution, and both *Centaurium* and *Cicendia* contain species in North, Central and temperate parts of South America, 2) the exact position of the neotropical Helieae sensu Gilg (clade G) within clade C is unresolved, and 3) the Central American *Geniostemon* is sister to clade I (Struwe et al., 1998b), a close relationship between *Ixanthus* and the neotropical *Symbalanthus* (clade D) and *Lisianthus* (clade E) as suggested by Meszaros et al. (1996) can be ruled out. This does not deny the existence of other direct Macaronesian/New World relationships which have been demonstrated for *Pericallis* D. Don (Kadereit and Jeffrey 1996) and some Crassulaceae (*Sedum nudum* Aiton and *S. fusiforme* Löwe; Van Ham and 'T Hart 1998).

It seems likely that the basal woodiness of *Ixanthus* is best interpreted as secondary. The wood of this species is initially almost rayless (Metcalf and Chalk 1950; Carlquist 1974, 1984). This and the presence of erect ray cells in the outer part of the wood have been

interpreted as evidence for secondary woodiness by Carlquist (1962, 1974, 1984). In clade I, *Ixanthus* and *Orphium* are basally woody, and basal woodiness is also found in some species of *Chironia* and *Eustoma*. This distribution of basal woodiness implies that a herbaceous vs. a basally woody habit are (depending on the resolution of the polytomy in clade I) more or less equally parsimonious as the plesiomorphic state of this clade.

Although the comparison with *Blackstonia* as a herbaceous taxon from open habitats might suggest that the woodiness of *Ixanthus* is related to the laurel forest habitat, there exist Macaronesian genera in which secondary woodiness does not appear to be primarily related to a laurel forest ecology. Thus *Echium*, *Isoplexis* and *Sonchus* all contain secondarily woody species which grow inside and outside the laurel forest (Hohenester and Welss 1993, Böhle et al. 1996, Kim et al. 1996, Carvalho and Culham, in press). In consequence, it seems more likely that woodiness in *Ixanthus* either evolved in response to its insular distribution (Carlquist 1974, Givnish et al. 1995) or simply is a consequence of its perennial life cycle.

The occurrence of *Ixanthus* in the Canary Islands provides no data to calibrate the ITS divergence between this species and *Blackstonia* because it can not be assumed that *Ixanthus* arose only after and immediately after the formation of these islands ca. 20 myrs ago (Coello et al. 1992). Accordingly, an estimate of the age of *Ixanthus* must rely on ITS divergence rates calculated for other taxa of similar habit and ecology. Estimations of ITS substitution rates are available for *Echium* from the Canary Islands (1.1×10^{-9} ; Böhle et al. 1996), for *Robinsonia* DC. (7.83×10^{-9} ; Sang et al. 1995) and *Dendroseris* D. Don (3.94×10^{-9} – 6.06×10^{-9} ; Sang et al. 1994) from the Juan Fernandez Islands, and for *Gossypium* L. (Wendel et al. 1995). Using these rates, an age of 6.6 to 47.1 myrs can be calculated for *Ixanthus*. This age range contains no information about the origin of

Ixanthus within or outside the Canary Islands. At the time of origin of *Ixanthus*, however, NW Africa and, after having come into existence, the Canary Islands, were dominated by laurel forests which were well developed between c. 45 and 3 myrs ago (Mai 1995).

In view of the observation that both *Ixanthus* and *Blackstonia* today grow in areas which were dominated by laurel forests when the two genera split, it can be asked whether the extant laurel forest habitat of *Ixanthus* is primary or secondary, i.e. whether the common ancestor of *Blackstonia* and *Ixanthus* grew in laurel forests or open habitats. Because all members (except for some species of *Sabatia*) of the *Ixanthus* clade (clade I) grow in open habitats, the application of a parsimony argument would favour an interpretation of the laurel forest habitat of *Ixanthus* as secondary. A parsimony argument, however, may not be decisive in the present context because the parallel evolution into open habitats of all relatives of *Ixanthus* is a realistic possibility when aridification was a general phenomenon in the northern hemisphere in the late Tertiary. Such widespread parallel evolution in this period has been hypothesized for, e.g. *Papaver* L. by Kadereit et al. (1997).

The only possible argument in favour of an interpretation of the laurel forest habitat as primary is the hypothesized time of origin of the genus because at this time laurel forests were widespread. This argument, however, is weakened by the observation that Tertiary laurel forest vegetation almost certainly will have contained open patches. In summary, the evidence for the interpretation of the laurel forest habitat as either primary or secondary is essentially inconclusive.

The above considerations illustrate that the interpretation of *Ixanthus* as a Tertiary relict (Meszaros et al. 1996) was misled by an erroneous assessment of its phylogenetic relationships. The recognition of its close relationship to *Blackstonia* in a mostly herbaceous clade together with the anatomical

evidence for the secondary character of its woodiness allows to interpret *Ixanthus* as a derived product of the radiation of a herbaceous group rather than as an impoverished relict taxon.

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