Cladogram of Panamanian *Clusia* Based on Nuclear DNA: Implications for the Origins of Crassulacean Acid Metabolism

H. H. Gehrig [1], J. Aranda [1], M. A. Cushman [2], A. Virgo [1], J. C. Cushman [2], B. E. Hammel [3], and K. Winter [1]

[1] Smithsonian Tropical Research Institute, Balboa, Ancon, Republic of Panama
[2] University of Nevada, Department of Biochemistry, MS 200, Reno, NV 89557, USA
[3] Missouri Botanical Garden, POB 299, St. Louis, MO 63166, USA

Received: November 14, 2002; Accepted: January 8, 2003

Abstract: **The internal transcribed spacer (ITS) region of 18-26S nuclear ribosomal DNA repeat was sequenced for 31 of the approximately 40 species of *Clusia* known to occur in Panama. Several species from other genera of the Clusiaceae were used as outgroups in the phylogenetic calculation. High sequence alignment and minimal length variation among ITS-1, 5.8S and ITS-2 sequences facilitated determination of positional homology of nucleotide sizes. Sequence alignment was evaluated with character state (Maximum Parsimony) and distance methods (Neighbour Joining). Phylogenetic trees obtained with the two methods were largely concordant and revealed three main groups that roughly correspond to previous arrangements of species into three large morphological groups, the *C. flava* group, the *C. minor* group and the *C. multiflora* group. Because species of *Clusia* are either regular C_3 plants or exhibit crassulacean acid metabolism (CAM) involving varying proportions of CO_2 fixation in the dark versus the light, we mapped photosynthetic pathways onto the cladograms. Photosynthetic pathway classification was based on measurements of $^{13}C/^{12}C$ ratios of plant carbon and also on information available from the literature. Both the *C. flava* and *C. minor* group contained species exhibiting CAM, distributed on distinct branches of the cladograms, whereas the third group (*C. multiflora* group) was composed of species which are not known to use CAM.**

Key words: ***Clusia*, Clusiaceae, internal transcribed spacer (ITS), molecular phylogeny, crassulacean acid metabolism (CAM).**

Abbreviations:

CAM:	crassulacean acid metabolism
ITS:	internal transcribed spacer
MP:	maximum parsimony
NJ:	neighbour joining
PCR:	polymerase chain reaction

Plant biol. 5 (2003) 59 – 70

ISSN 1435-8603

Introduction

The neotropical genus *Clusia* comprises an estimated 300 species of woody plants (Pipoly et al., 1998) which are either terrestrial trees, epiphytes, hemiepiphytes or, occasionally, lianas and which occur in a large range of habitats, such as lowland and montane forests, savannas or coastal sand dunes (Lüttge, 1991). *Clusia* taxonomy has been notoriously difficult to study, given only morphological data (Hammel, 1986; Pipoly et al., 1998). Flowers are present for only short periods and do not retain their character well in herbarium collections. Morphological distinctions based on fruit and vegetative materials can be ambiguous (Pipoly et al., 1998). Recent advances in the use of molecular phylogenetic techniques will undoubtedly help to resolve some taxonomic uncertainties. The chloroplast gene *rbc*L has been sequenced for species of all genera of the Clusiaceae (Gustafsson et al., 2002). ITS data of ribosomal DNA have been published for several species of *Clusia* (Vaasen et al., 2002).

The diversity of life forms and habitats of the genus *Clusia* is matched by a remarkable diversity in photosynthetic physiology because some species of *Clusia* exhibit crassulacean acid metabolism (CAM) (Winter and Smith, 1996), a mode of carbon assimilation not commonly associated with trees (Lüttge, 1996; Ting et al., 1985; Tinoco Ojanguren and Vásquez-Yanes, 1983). CAM is characterized by CO_2 uptake at night and represents a mechanism that improves the ability of land plants to assimilate carbon in water-limited environments. In *Clusia,* CAM may be strongly *(Clusia rosea)* or weakly expressed *(C. minor),* and certain species, such as *C. minor* or *C. uvitana,* show considerable plasticity in the expression of CAM in relation to environmental stress as their rates of dark CO_2 fixation may strongly and reversibly increase in response to water shortage (Borland and Griffiths, 1996; Winter et al., 1992; Zotz and Winter, 1993, 1994 a, b). Only a small number of *Clusia* species have been screened for their photosynthetic pathway to date (Lüttge, 1999). Therefore, the exact proportion of C_3 versus CAM species is not known. Many species of *Clusia* have $\delta^{13}C$ values indicative of C_3 photosynthesis but this does not exclude the possibility that small portions of daily carbon gain may be derived from dark CO_2 fixation via CAM (Holtum and Winter, 1999; Pierce et al., 2002; Winter and Holtum, 2002). However, the notion that all species of *Clusia* may have at least some potential to exhibit CAM does not appear to be correct. Taxa belonging to the *C. multiflora* species complex, for exam-

ple, are probably constitutive C_3 plants; thorough studies failed to reveal any signs of CAM in such material (Grams et al., 1998).

Given that the genus *Clusia* contains both C_3 and CAM species, a study of phylogenetic relationships among these species seems to be a promising approach to learn more about the evolutionary origins of the CAM pathway. Thus far, robust molecular phylogenies have been successfully established for only two taxa tracing the origins of CAM: the genus *Kalanchoe* (Crassulaceae) (Gehrig et al., 2001) and the family Bromeliaceae (D. Crayn, K. Winter, J. A. C. Smith, in preparation). Interpretation of data is complicated for *Kalanchoe* because essentially all species have the capacity for CAM. In the Bromeliaceae, which, like *Clusia,* contain both C_3 and CAM species, CAM evolved independently at least three times (D. Crayn, K. Winter, J. A. C. Smith, unpublished data).

One previous attempt was made to link phylogenetic relationships and the occurrence of CAM in *Clusia* (Vaasen et al., 2002). In that study, ITS sequences were analyzed in order to construct a phylogenetic tree for 17 species of *Clusia* from live collections in various botanical gardens in Germany. Little agreement was found between species clusters from molecular data and previous groupings based on morphological characteristics. Furthermore, there was no indication for group-specific evolution of CAM.

Because increased sample size will be necessary in order to unravel phylogenetic relationships within *Clusia,* and as part of ongoing research on the ecology and physiology of Panamanian species of *Clusia,* we have investigated the molecular phylogeny of 31 of the approximately 40 Panamanian species based on nucleotide sequences of the ITS-1 and ITS-2 regions. We constructed a phylogenetic tree on which the absence or presence of CAM, as determined from carbon isotope ratios and, if available, from literature data on net CO_2 exchange characteristics and diel changes in organic acid content, was mapped.

Materials and Methods

Plant material

Plant material was collected during several field trips to different provinces of the Republic of Panama. Specimens were deposited at the Smithsonian Tropical Research Institute (Table **1**). With few exceptions, several individuals from each species were collected at various locations and sequenced. Voucher specimens in Table **1** refer to the plant material used for construction of phylogenetic trees that was selected at random from individuals identified by one of us (B.E.H.).

DNA isolation, PCR amplification and purification

Fresh leaves were frozen in liquid nitrogen and stored at – 90 °C. Total DNA was extracted from 250 to 300 mg tissue using the Dneasy® Plant Mini Kit (Qiagen, Valencia, CA, USA) following the manufacturer's instructions. DNA was stored at + 4 °C if not used directly. The entire internal transcribed spacer region, comprising ITS-1, the 5.8S gene and ITS-2, was amplified via the polymerase chain reaction (PCR) in 100 µl volumes of 10 mM Tris buffer (pH 8.5) containing 50 mM KCl, 2 mM $MgCl_2$, 0.2 mM of dNTP mix, 25 µM of each primer, 0.1 to 0.5 µg of template DNA, and 2.5 units of Taq polymerase using the external "ITS-1" and "ITS-4" primers designed by White et al. (1990). PCR was performed in a Perkin Elmer Thermal Cycler (Perkin Elmer, Instrument Division Norwalk, CT, USA) after an initial denaturation step of 5 min at 94 °C via 35 cycles of 1 min denaturation at 94 °C, 1 min at 52 °C for primer annealing and 2 min of extension at 72 °C for each cycle. A 10-min final extension at 72 °C followed cycle 35.

Five µl of each PCR reaction product were separated on a 1.2 % agarose gel using 1 × TAE as the gel buffer, to assess the quality of amplification. Successful PCR amplifications resulted in a single DNA band corresponding to the ~ 700 bp marker. ITS fragments were purified with the MinElute PCR Purification Kit (Qiagen).

Two to 3.5 µl of the purified PCR fragments were ligated into pCR 2.1-TOPO® vector or pCR II-TOPO® vector from Invitrogen™ Life Technologies Corporation (Carlsbad, CA, USA). One µl of the 3 h or overnight ligation product was transformed in TOP 10 cells (Invitrogen). Plasmid DNA was isolated after the alkaline lysis Miniprep method (STETL protocol [Saccharose, Tris, EDTA, Triton, Lysozym]) of Sambrook and Russell (2001) and sequenced (some in both directions) on a Perkin-Elmer Applied Biosystems 3700 automated DNA sequencing system (Nevada Genomics Center, University of Nevada, Reno) using the Prism™ Ready Reaction Dyedeoxy™ Terminator Cycle Sequencing kit (Perkin-Elmer, Applied Biosystems Division, USA).

Sequence alignment and analysis

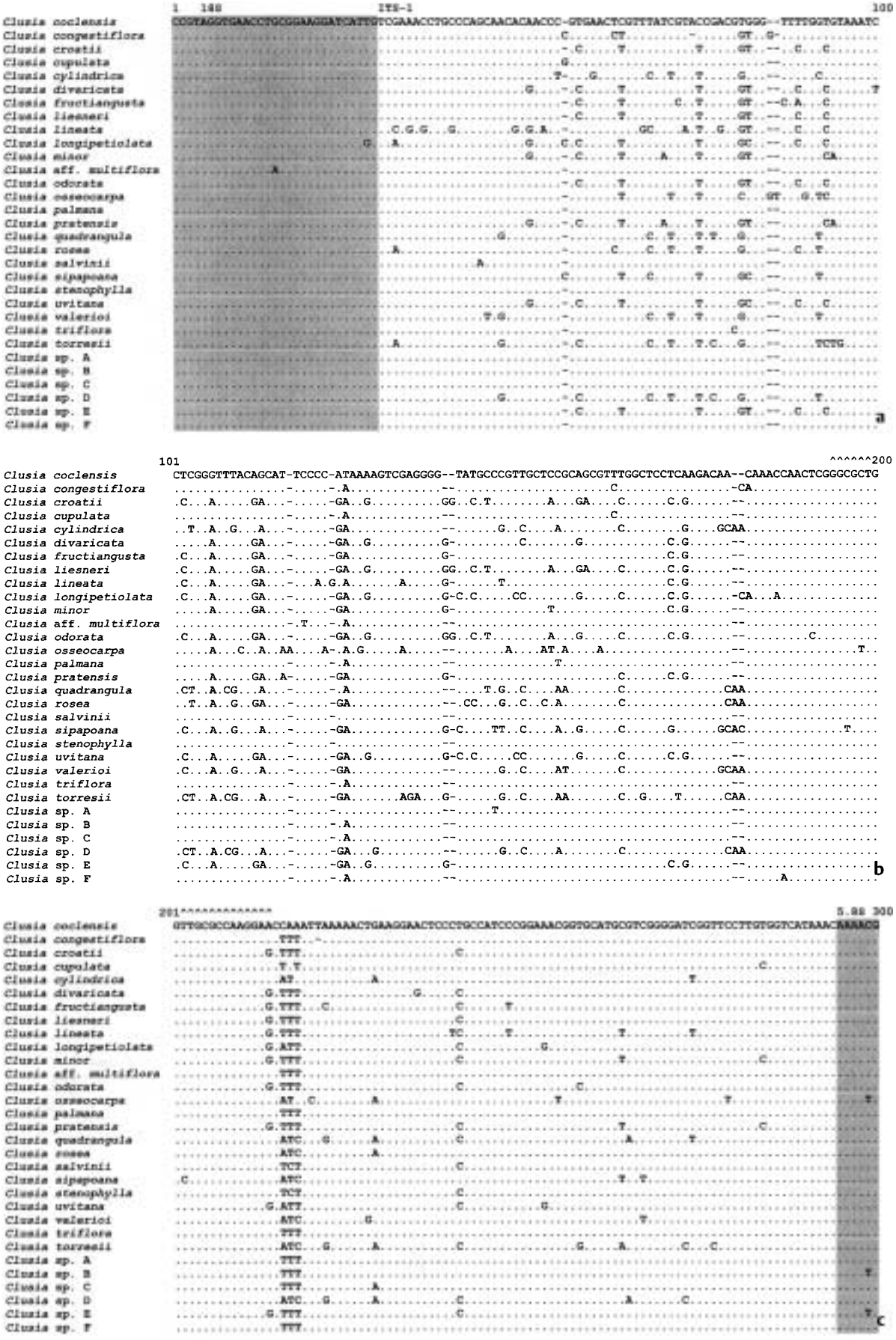
The alignment of ITS sequences was calculated for each pair using the program ALIGN 4.0 (demonstration version for Windows 95/98/NT: http://www-3.igb-berlin.de/abt3/hepperle/index.html). The alignment obtained was modified by visual inspection to increase the total alignment score. Alignment for Panamanian species of *Clusia* is shown in Figs. **1 a – g**. Aligned data for other Clusiaceae sequenced here can be obtained from the first author (hansgehrig@gmx.de). The boundaries of ITS-1, ITS-2 and 5.8S rRNA were determined by comparison with various published sequences available in GenBank® NCBI (National Center for Biotechnology Information; http://www.ncbi.nlm.nih.gov/Genbank/index.html).

Numbers and proportions of nucleotide site divergence were calculated for all possible pairwise comparisons of ITS-1 and ITS-2 and the combined ITS-1 and ITS-2 sequence data. Only the sites without gap and nucleotide ambiguity were included in the comparisons. The formula $P = N_d / (N_d + N_I) \times 100\,\%$ was used for calculation, where P is the percentage of site divergence, N_d is the number of divergent nucleotide sites and N_I is the number of identical nucleotide sites shared by the two sequences compared. Three species of the genus *Calophyllum* (Clusiaceae, Kielmeyeroideae) were selected as an outgroup. Several other species of Clusiaceae (representing Symphonieae and Garcinieae) were included in the phylogenetic comparison.

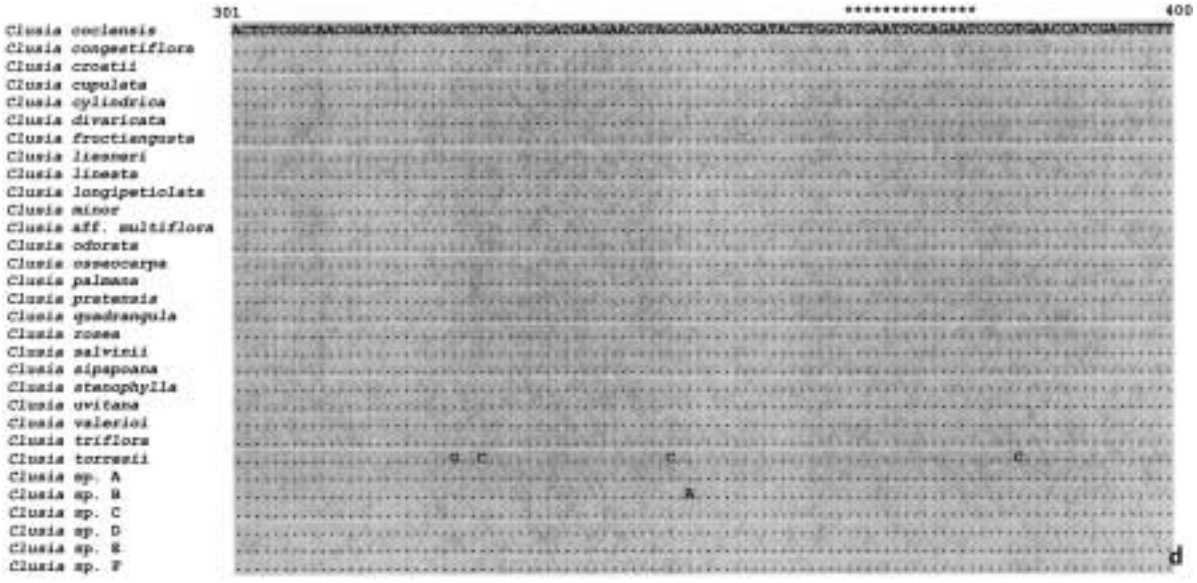
Table 1 Voucher specimens for ITS analyses of Panamanian Clusiaceae that were used to establish the phylogenetic relationships shown in Figs. **2** and **3**. Collections were made by Aranda et al. and are deposited at the Smithsonian Tropical Research Institute (Tupper Center), Republic of Panama. (BO) Bocas del Toro, (CC) Cocle, (CH) Chiriqui, (CO) Colon, (PA) Panama, (VR) Veraguas. (?) denotes tentative identification by B. E. Hammel

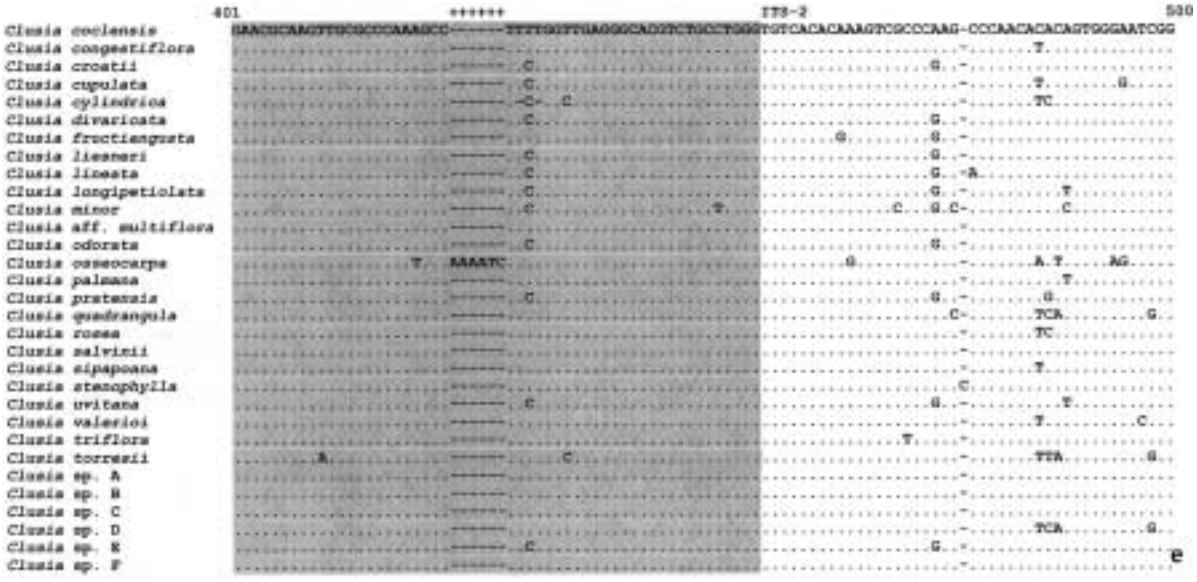
Species	Locality, Province	Voucher	GenBank accession no.
Clusia coclensis Standl. ?	Cerro Colorado (CH)	3813	AJ509187
Clusia congestiflora Cuatrec.	El Llano-Cartí (PA)	3711	AJ509206
Clusia croatii D'Arcy	Changuinola (BO)	3759	AJ509186
Clusia cupulata (Maguire) Maguire	El Llano-Cartí (PA)	3712	AJ509195
Clusia cylindrica Hammel	Almirante (BO)	3774	AJ509201
Clusia divaricata Maguire	El Valle (CC)	3653	AJ509192
Clusia fructiangusta Cuatrec.	El Llano-Cartí (PA)	3691	AJ312575
Clusia liesneri Maguire	Cerro Jefe (PA)	3755	AJ509185
Clusia lineata (Benth.) Planch. and Triana	El Copé (CC)	3655	AJ312556
Clusia longipetiolata Schery	Changuinola (BO)	3772	AJ509190
Clusia minor L.	Río Sereno (CH)	3829	AJ509208
Clusia aff. *multiflora* Kunth	Cerro Jefe (PA)	3700	AJ312588
Clusia odorata Seem.	Boquete (CH)	3794	AJ509188
Clusia osseocarpa Maguire	Cerro Jefe (PA)	3850	AJ509193
Clusia palmana Standl.	Fortuna (CH)	3789	AJ509198
Clusia pratensis Seem.	El Valle (CC)	3621	AJ509196
Clusia quadrangula Bartlett	Guabal (VR)	3840	AJ509204
Clusia rosea Jacq.	El Valle (CC)	3724	AJ509203
Clusia salvinii Donn. Sm.	Santa Fé (VR)	3739	AJ509197
Clusia sipapoana (Maguire) Pipoly	Altos de Pacora (PA)	3668	AJ312562
Clusia stenophylla Standl.	El Valle (CC)	3612	AJ509184
Clusia torresii Standl.	Cerro Colorado (CH)	3801	AJ509202
Clusia triflora Cuatrec. ?	Cerro Colorado (CH)	3804	AJ509207
Clusia uvitana Pittier	Portobelo (CO)	3845	AJ509226
Clusia valerioi Standl.	Portobelo (CO)	3844	AJ509200
Clusia sp. A	Altos de Pacora (PA)	3631	AJ312548
Clusia sp. B	Cerro Colorado (CH)	3854	AJ509199
Clusia sp. C	Cerro Colorado (CH)	3849	AJ509191
Clusia sp. D	Río Sereno (CH)	3831	AJ509205
Clusia sp. E	Campana (PA)	3599	AJ312541
Clusia sp. F	El Llano-Cartí (PA)	3833	AJ509194
Calophyllum inophyllum L.	Campana (PA)	4101	AJ312608
Calophyllum longifolium Willd.	Campana (PA)	4100	AJ312609
Calophyllum nubicola D'Arcy and R. C. Keating	Cerro Jefe (PA)	3754	AJ312610
Chrysochlamys eclipes L. O. Williams	El Llano-Cartí (PA)	3746	AJ509212
Chrysochlamys glauca (Oerst. ex Planch. and Triana) Hemsl.	Río Sereno (CH)	3827	AJ509213
Chrysochlamys grandifolia (L. O. Williams) Hammel	Campana (PA)	3593	AJ509209
Chrysochlamys skutchii Hammel	Santa Fé (VR)	3838	AJ509211
Chrysochlamys tenuis Hammel	Campana (PA)	3595	AJ509210
Garcinia madruno (Kunth) Hammel	Santa Fé (VR)	3735	AJ509215
Garcinia mangostana L. f.	Gamboa (PA)	3856	AJ509214
Garcinia sp.	El Copé (CC)	3660	AJ312607
Garcinia intermedia (Pittier) Hammel	Cerro Jefe (PA)	3649	AJ312605
Symphonia globulifera L.	Campana (PA)	3594	AJ312606
Dystovomita paniculata (Donn. Sm.) Hammel	El Valle (CC)	3723	AJ509216
Tovomita longifolia (Rich.) Hochr.	El Llano-Cartí (PA)	3710	AJ312591
Tovomita weddelliana Planch. and Triana	Cerro Jefe (PA)	3642	AJ509218

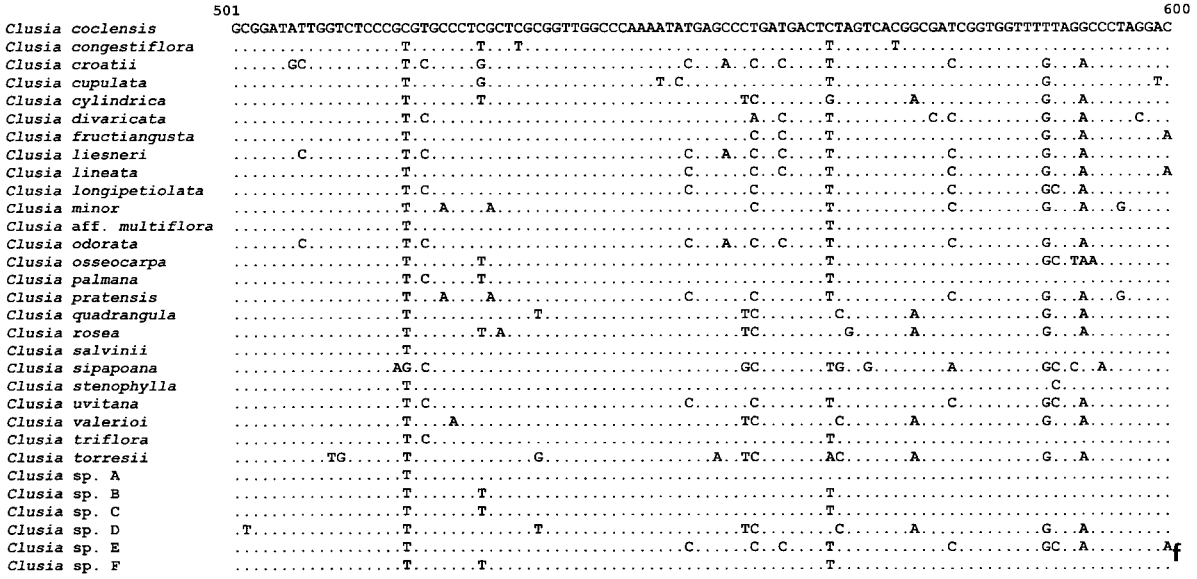
Figs. 1 a – c


1 100
ITS-1
Clusia coclensis
Clusia congestiflora
Clusia croatii
Clusia cupulata
Clusia cylindrica
Clusia divaricata
Clusia fructiangusta
Clusia liesneri
Clusia lineata
Clusia longipetiolata
Clusia minor
Clusia aff. multiflora
Clusia odorata
Clusia osseocarpa
Clusia palmana
Clusia pratensis
Clusia quadrangula
Clusia rosea
Clusia salvinii
Clusia sipapoana
Clusia stenophylla
Clusia uvitana
Clusia valerioi
Clusia triflora
Clusia torresii
Clusia sp. A
Clusia sp. B
Clusia sp. C
Clusia sp. D
Clusia sp. E
Clusia sp. F
a
101 ^^^^^^200
Clusia coclensis CTCGGGTTTACAGCAT-TCCCC-ATAAAAGTCGAGGGG--TATGCCCGTTGCTCCGCAGCGTTTGGCTCCTCAAGACAA--CAAACCAACTCGGGCGCTG
b
201^^^^^^^^^^^^^^ 5.8S 300
Clusia coclensis GTTGCGCCAAGGAACCAAATTAAAAACTGAAGGAACTCCCTGCCATCCCGGAAACGGTGCATGCGTCGGGGATCGGTTCCTTGTGGTCATAAACAAAACG
c

Figs. 1 d – f


301
400
Clusia coclensis
Clusia congestiflora
Clusia croatii
Clusia cupulata
Clusia cylindrica
Clusia divaricata
Clusia fructiangusta
Clusia liesneri
Clusia lineata
Clusia longipetiolata
Clusia minor
Clusia aff. multiflora
Clusia odorata
Clusia osseocarpa
Clusia palmana
Clusia pratensis
Clusia quadrangula
Clusia rosea
Clusia salvinii
Clusia sipapoana
Clusia stenophylla
Clusia uvitana
Clusia valerioi
Clusia triflora
Clusia torresii
Clusia sp. A
Clusia sp. B
Clusia sp. C
Clusia sp. D
Clusia sp. E
Clusia sp. F
d


401
500
ITS-2
Clusia coclensis
Clusia congestiflora
Clusia croatii
Clusia cupulata
Clusia cylindrica
Clusia divaricata
Clusia fructiangusta
Clusia liesneri
Clusia lineata
Clusia longipetiolata
Clusia minor
Clusia aff. multiflora
Clusia odorata
Clusia osseocarpa
Clusia palmana
Clusia pratensis
Clusia quadrangula
Clusia rosea
Clusia salvinii
Clusia sipapoana
Clusia stenophylla
Clusia uvitana
Clusia valerioi
Clusia triflora
Clusia torresii
Clusia sp. A
Clusia sp. B
Clusia sp. C
Clusia sp. D
Clusia sp. E
Clusia sp. F
e


501
600
Clusia coclensis GCGGATATTGGTCTCCCGCGTGCCCTCGCTCGCGGTTGGCCCAAAATATGAGCCCTGATGACTCTAGTCACGGCGATCGGTGGTTTTTAGGCCCTAGGAC
Clusia congestiflora
Clusia croatii
Clusia cupulata
Clusia cylindrica
Clusia divaricata
Clusia fructiangusta
Clusia liesneri
Clusia lineata
Clusia longipetiolata
Clusia minor
Clusia aff. multiflora
Clusia odorata
Clusia osseocarpa
Clusia palmana
Clusia pratensis
Clusia quadrangula
Clusia rosea
Clusia salvinii
Clusia sipapoana
Clusia stenophylla
Clusia uvitana
Clusia valerioi
Clusia triflora
Clusia torresii
Clusia sp. A
Clusia sp. B
Clusia sp. C
Clusia sp. D
Clusia sp. E
Clusia sp. F
f

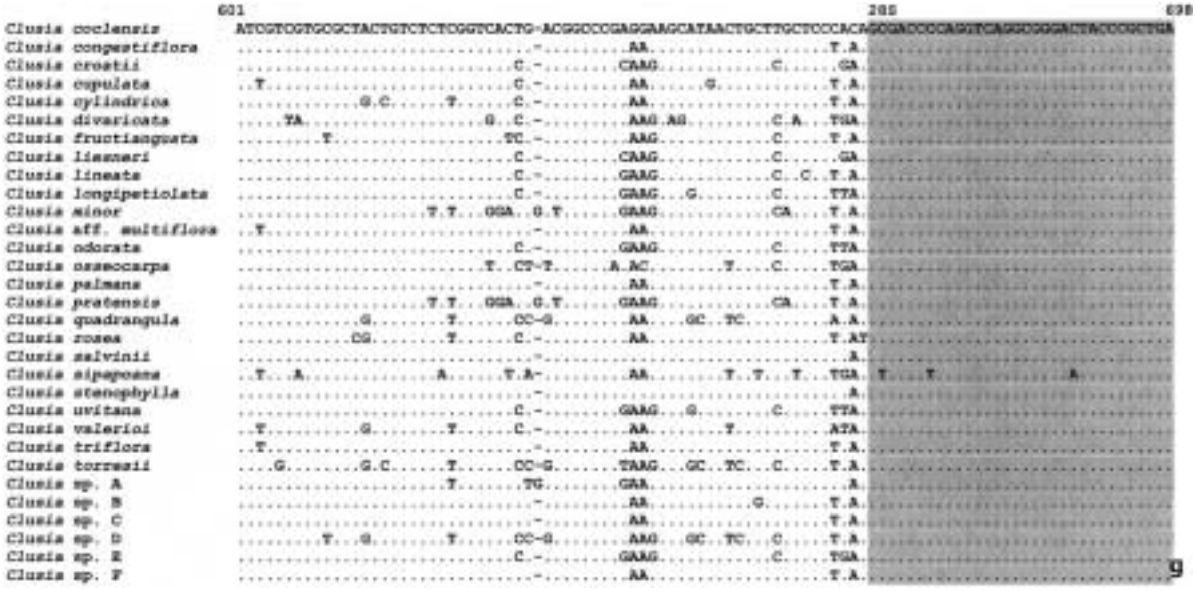


Fig. 1 g

Figs. 1 a – g Aligned ITS sequences from 31 Panamanian species of *Clusia.* Gaps are indicated by "–", and the coding regions for the ribosomal DNA by a grey background. "^" indicates a conserved motif in the ITS-1 region, and "∗" a conserved motif in the 5.8S rDNA gene. "+" indicates an insertion of 6 bp in the 5.8 rDNA gene of *Clusia osseocarpa.*

Nucleotide sites from ITS-1, 5.8S and ITS-2 sequences with potential phylogenetic information, i.e., with at least two nucleotide states, each present in at least two sequences; were included in a data matrix. Only nucleotide sites with unambiguous alignment were used in the phylogenetic analysis. Gaps were treated as missing data. The resulting matrix was analyzed using the distance method Neighbour Joining (NJ, as a tool in the program package PHYLP, version 3.4; Felsenstein, 1993) and the Maximum Parsimony method (MP; phylogeny program version 4.0b8; Swofford, 2002). Sequence divergence values (NJ) between species were calculated after bootstrapping (1000 replicates) by the two-parameter method (Kimura, 1980) using the DNADIST program of PHYLIP. This method allows for the correction of multiple substitutions and differential transition/transversion probabilities based on empirical observation from the data. The ratio was set at 1.0, based on the actually observed frequencies (Kimura, 1980) in the maximum parsimony tree. As a result, it was assumed that there is an equal probability of independent change at all sites (Jukes and Cantor, 1969).

Heuristic searches (PAUP: MULTIPARS option) and the test for maximum parsimony were carried out in two steps. In a first step, the matrix was searched for different islands using 5000 random additions without swapping. Then the trees were optimized with the TBR swapping procedure. Clade support was assessed by bootstrap (1000 replicates and with the "fast stepwise addition" function). All characters were treated as unordered and given equal weight. Alignment gaps were treated either as missing data, or as a fifth character when the number of deleted bases did not exceed 2 bp. Trees were oriented with three species of *Calophyllum* (Clusiaceae, Kielmeyeroideae) as the functional outgroup. All most parsimonious trees were saved.

δ13C analyses

Carbon isotope ratios were determined for CO_2 derived from 3-mg samples of dried herbarium tissue (mature leaves) (Crayn et al., 2001; Winter and Holtum, 2002). Samples were analyzed with a mass spectrometer at the Analytical Chemistry Laboratory, Institute of Ecology, University of Georgia, Athens. The abundance of ^{13}C in each sample was calculated relative to the abundance of ^{13}C in standard CO_2 calibrated against the Pee Dee belemnite *(Belemnitella americana).* Relative abundance was determined using the relationship

$$\delta^{13}C\,(‰) = [(^{13}C/^{12}C \text{ of sample})/(^{13}C/^{12}C \text{ of standard}) - 1] \times 1000.$$

Results

Characteristics of the ITS region

One or three positive clones of a ~ 0.7-kb cDNA fragment from each of the species listed in Table **1** were completely sequenced in both directions, covering the 3′ end of 18S rRNA (position 1 – 29 in the alignment), the first internal transcribed spacer ITS-1 (position 30 – 292), the 5.8S rRNA (position 293 – 452), the second internal transcribed spacer ITS-2 (position 453 – 661) and the 5′ end of the 26S rRNA (position 662 – 698). The length range of the different regions and G + C content of each sequence are given in Table **2**. Out of the 698 bp aligned positions in the entire fragment length, 19 sites involve gaps (10 in ITS-1, 6 in 5.8S rRNA, 3 in ITS-2). The 5.8S rRNA gene region is highly conserved in all species; only four single point mutations could be detected. The six gaps found in the 5.8S gene region are a result of an insertion (AAAATC) in the sequence of *Clusia osseocarpa,* between position 420 – 425 in the alignment (Figs. **1 a – g**). Two conserved angiosperm motifs were detected: one in the ITS-1 spacer region between position 192 – 212 (5′-GGC GCT GGT TGC GCC AAG GAA-3′), and

Table 2 Characteristics and variations of the ITS nrDNA region of Panamanian Clusiaceae

Sequence characteristics	ITS-1	ITS-2	ITS-1 + ITS-2	5.8S
Length range (bp)				
– ingroup	254 – 260	205 – 209	461 – 472	156 – 162
– outgroup	263	217	480	162
% of G + C content	48.0 – 58.1	48.3 – 61.1	48.9 – 59.8	49.7 – 53.9
Invariant characters	173	151	324	148
Variable characters	74	48	128	14
Potentially informative characters	34	21	55	7
Proportion (%) of nucleotide differences between pairs of species				
Within genus *Clusia*	1.4 – 18.5	0.2 – 18.2	1.4 – 15.4	0 – 2.6
Between genus *Clusia* and other Clusiaceae	9.7 – 47.3	2.8 – 44.1	1.3 – 44.7	0 – 14.6

the second inside the 5.8S rRNA gene region between position 465 – 478 (5′-GAA TTG CAG AAT TCC-3′). Both motifs were previously identified in angiosperms and have been used to differentiate between flowering plants, fungi and algae (Liu and Schardl, 1994; Jobes and Thien, 1997).

Pairwise comparisons between all possible combinations were carried out for ITS-1, ITS-2 and for the combined ITS-1/ITS-2 regions (distance matrices not shown). Ambiguous and gap sites were excluded from these comparisons. Sequence divergence between pairs of *Clusia* species ranged from 1.4% (*C. salvinii* and *C.* sp. B) to 18.5% (*C. lineata* and *C. congestiflora*) for ITS-1, from 0.2% (*C. uvitana* and *C. longipetiolata*) to 18.2% (*C. minor* and *C. sipapoana*) for ITS-2 and from 1.4% (*C. salvinii* and *C. palmana*) to 15.4% (*C. odorata* and *C. torresii*) for ITS-1/ITS-2. Between the *Clusia* species and the chosen outgroup species (*Calophyllum* ssp.) the corresponding values for ITS-1 ranged from 42.8% *(C. coclensis)* to 47.3% *(C. lineata),* for ITS-2 from 39.6% *(C. stenophylla)* to 46.2% *(C. sipapoana)* and for ITS-1/ITS-2 from 41.9% *(C. coclensis)* to 46% *(C. sipapoana).* These data refer to individuals selected for construction of phylogenetic trees (See "Materials and Methods" section and Table **1**). In most of the 31 Panamanian species of *Clusia,* material was sequenced for up to 17 different individuals per species from various locations. ITS sequences of each species showed homology variation between 0.3 and 5.1% (Table **3**).

Table 3 Intraspecific variation in species of *Clusia* from different locations in Panama

Species	Individuals	Sequence variation (%)
Clusia coclensis ?	3	0.8 – 2.3
Clusia congestiflora	1	–
Clusia croatii	11	0.8 – 4.2
Clusia cupulata	2	3.2
Clusia cylindrica	5	2.8 – 4.8
Clusia divaricata	5	0.7 – 3.2
Clusia fructiangusta	4	1.7 – 4.8
Clusia liesneri	17	0.3 – 4.9
Clusia lineata	2	3.3
Clusia longipetiolata	4	1.6 – 4.9
Clusia minor	1	–
Clusia aff. *multiflora*	8	0.4 – 4.8
Clusia odorata	4	1.2 – 3.8
Clusia osseocarpa	5	0.5 – 4.8
Clusia palmana	8	1.1 – 5.1
Clusia pratensis	6	1.8 – 4.4
Clusia quadrangula	2	3.7
Clusia rosea	6	1.8 – 4.8
Clusia salvinii	3	2.6 – 3.2
Clusia sipapoana	1	–
Clusia stenophylla	5	0.5 – 3.1
Clusia torresii	4	2.7 – 3.5
Clusia triflora ?	1	–
Clusia uvitana	8	0.4 – 4.6
Clusia valerioi	11	1.1 – 5.1
Clusia sp. A	3	1.5 – 4.7
Clusia sp. B	2	1.5
Clusia sp. C	2	0.9
Clusia sp. D	1	–
Clusia sp. E	7	0.9 – 4.7
Clusia sp. F	4	1.5 – 4.0

Phylogenetic analyses of ITS sequences

Phylogenetic relationships derived from Neighbour Joining (NJ) analyses are shown in Fig. **2** and the strict consensus Maximum Parsimony (MP) tree is shown in Fig. **3**. Of 712 total characters included in the alignment, 388 are uninformative and 324 are parsimony informative. Note that less than 712 characters are shown in Figs. **1 a – g** because these data only refer to species of *Clusia.* The tree search yielded 162 453 most parsimonious trees (after condensing) with a length of 1155 steps. Consistency index (CI) was 0.5472, homoplasy index (HI) was 0.4528, and retention index (RI) was 0.7709 (CI excluding uninformative characters = 0.5014; HI excluding uninformative characters = 0.4986).

The two types of analysis result in very similar topologies. In both trees, the Clusioideae are strongly supported as monophyletic (100 and 98% bootstraps, respectively). They form three main branches: the Symphonieae, the Garcinieae and the Clusieae (100% bootstraps). Inside the Clusieae clade, the genus *Clusia* forms the monophyletic sister group to *Chrysochlamys* and *Tovomita.* The genus *Clusia* is separated into three main groups (Figs. **2, 3**), by and large corresponding to three morphological groups distinguished in earlier studies (Ham-

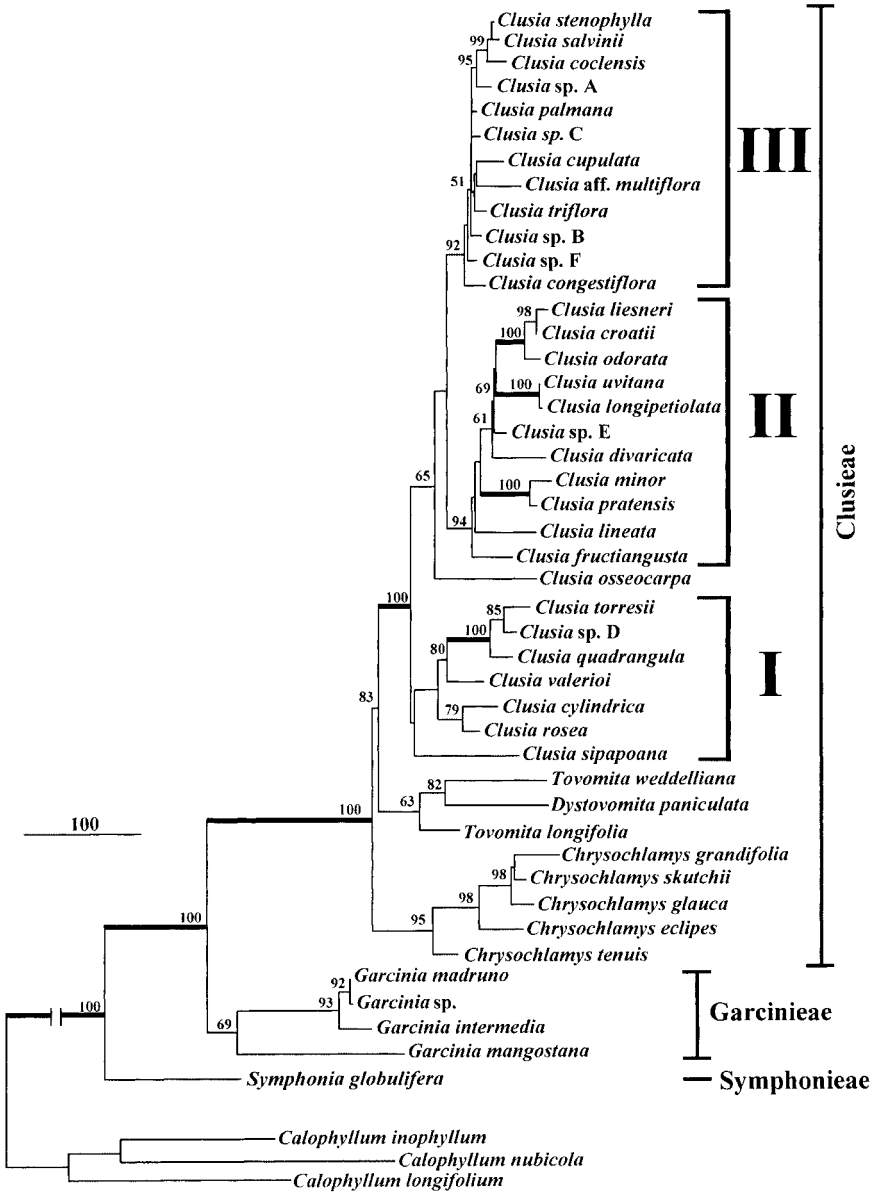


Fig. 2 Phylogenetic tree derived from Neighbour Joining analysis of a 698 bp alignment of ITS sequences of the nuclear rDNA, rooted with three species of *Calophyllum* (Clusiaceae). Bootstrap values (1000 replicates) below 50% are not shown. The branch length of the outgroup was shortened 1 : 10.

mel, 1986). The relationships of these three groups are weakly to well supported in both trees. Group I is a clade with 82% bootstrap support in the MP tree. Group II is supported by 94% in the NJ tree and 89% in the MP tree, and group III is supported by 92% in both the NJ tree and MP tree. Each of these three main groups contains up to three subgroups including distinct monophyletic branches in I and II. Two branches in group I are represented by *C. rosea* + *C. cylindrica* and the monophyletic *C. quadrangula* + *C. torressii* + *C.* sp. D. In group II, the branches consisting of *C. minor* + *C. pratensis, C. uvitana* + *C. longipetiolata* and *C. odorata* + *C. liesneri* + *C. croatii,* can be considered monophyletic, with bootstrap values of 100% in NJ and MP trees, except for the latter branch which shows bootstrap values of 100% in the NJ tree and 86% in the MP tree. Other *Clusia* groupings in I to III are poorly supported (bootstrap values below 50% are not given). *Clusia osseocarpa* is located between I and II in both trees, supported by 84% in the MP tree and 65% in the NJ tree.

Occurrence of CAM

A survey of $\delta^{13}C$ values of *Clusia* plants used for our ITS analysis showed that only two species in our plant collection had $\delta^{13}C$ values suggesting substantial dark CO_2 fixation via the CAM pathway: *C. rosea,* – 19.9‰; *C. uvitana,* – 19.7‰. The vast majority of *Clusia* species studied here (29 out of 31) had $\delta^{13}C$ values between – 24.0 and – 30.4‰, with a mean of – 27.0‰ (SD ± 1.6‰), indicating that most if not all carbon of these species was derived via C_3 photosynthesis in the light. (The complete set of $\delta^{13}C$ values determined for species studied here will be published elsewhere [K. Winter et al., in preparation].) However, $\delta^{13}C$ analysis does not distinguish among species showing exclusively C_3 photosynthesis and species that fix small portions of CO_2 via CAM in the dark. *C. minor,* although possessing a C_3 type $\delta^{13}C$ value of – 27‰ (see also Diaz et al., 1996; Franco et al., 1994) has been shown in numerous studies to exhibit CAM under conditions of drought and high irradi-

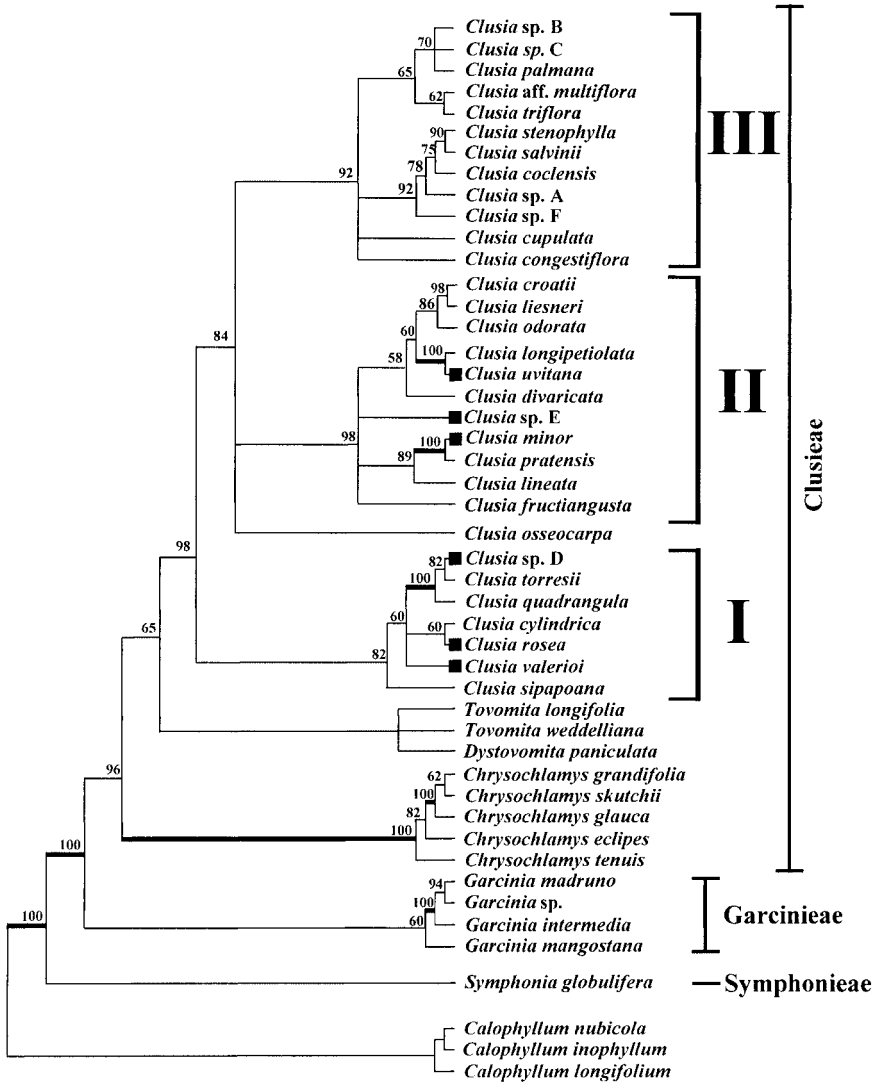


Fig. 3 Strict consensus tree of the most parsimonious trees found by heuristic analysis of ITS sequences of the nuclear rDNA, rooted with three species of *Calophyllum* (Clusiaceae). Bootstrap values (1000 replicates branch-and-bound Wagner analyses) below 50% are not shown. (■) indicates species known to exhibit crassulacean acid metabolism.

ance levels (Borland and Griffiths, 1996; Grams and Thiel, 2002) and was therefore considered a CAM species in our analysis. Evidently, *C. valerioi, C.* sp. D and *C.* sp. E also have the ability to perform a low degree of CAM, since small nocturnal increases in titratable acidity were recently reported for these species (Wanek et al., 2002). When photosynthetic pathway is mapped on the phylogenetic tree in Fig. **3**, species with CAM occur in group I (*C. rosea, C. valerioi, C.* sp. D) and group II (*C. minor, C. uvitana, C.* sp. E).

CAM has not been demonstrated for any of the species in group III, to date.

Discussion

ITS sequence comparison

ITS-1 regions were longer than ITS-2 regions. Among the ingroup taxa studied, length variation of DNA sequences ranged from 254 to 260 bp for ITS-1 and from 205 to 209 bp for ITS-2. The corresponding sequences in the outgroup contained 263 bp (ITS-1) and 217 bp (ITS-2). Similar length differences between ITS-1 and ITS-2 were found for many other angiosperm families (Valiejo-Roman et al., 2002). In a few plant families, such as the Gentianaceae (Yuan and Küpfer, 1995), the two ITS regions do not differ in length.

NJ and MP calculations resulted in well-supported cladograms with highly similar grouping of species. The intraspecific sequence variation, usually below 5%, as shown in Table **3** is at the higher end of that of other vascular plant taxa (Baldwin et al., 1995; Mayol and Rossello, 2001). This intraspecific sequence variation had no influence on the species arrangement. In both cladograms, for example, the position of *C. liesneri* did not change when calculations were done separately with each of the 17 sequences available for that species. The position of *C. liesneri* also remained unchanged when a consensus sequence of all 17 individuals was used for tree construction. Furthermore, construction of a NJ tree using all available sequences for all species, showed that individuals from each species clustered together on distinct branches on the cladogram (data not shown). We conclude that ITS sequences are useful markers for a phylogenetic interpretation of the Clusiaceae. Future studies must show whether some of the intraspecific sequence varia-

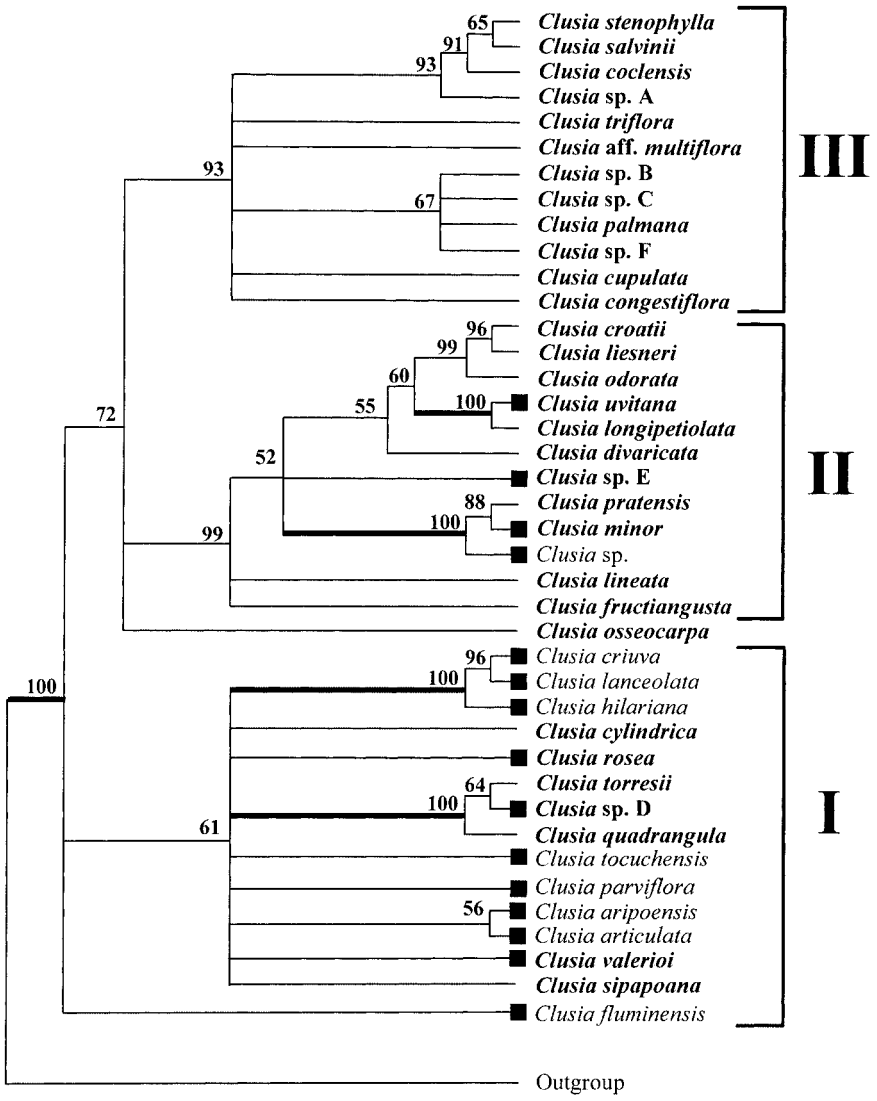


Fig. 4 Consensus tree derived from Neighbour Joining analysis (1000 bootstraps) of ITS sequences of *Clusia*, rooted with three species of *Calophyllum*, *Dystovomita grandifolia*, *Chrysochlamys skutchii*, *Garcinia madruno* and *Symphonia globulifera* as outgroup. The tree includes non-Panamanian species reported to exhibit CAM from a previous study (Vaasen et al., 2002) (names not in bold). *Clusia* sp., a CAM species in the branch containing *C. minor* and *C. pratensis* in group II, corresponds to material called "*C. alata*" in Vaasen et al. (2002). This material may have been misidentified in the previous study because *C. alata* almost certainly is a member of the *C. multiflora* group (equivalent to group III) and not of the *C. minor* group (equivalent to group II). (■) indicates species known to exhibit crassulacean acid metabolism.

tion is informative for resolving relationships among subspecies and/or indicates independent evolutionary histories of the *Clusia* populations sampled.

Molecular versus morphological groupings

A broad, integrated assessment of the phylogeny of *Clusia,* based on molecular and morphological data, does not yet exist. More than 350 species of *Clusia* have been described in the literature, but a substantial number of "species" may be synonyms (Pipoly and Graff, 1995). Most descriptions of *Clusia* are limited to specific areas (e.g., D'Arcy, 1980; Maguire, 1979; Pipoly et al., 1998). Our results suggest that the genus is monophyletic, forming a sister group to the genera *Chrysochlamys* and *Tovomita.* Such a sister group relationship was also shown by Gustafsson et al. (2002) who studied the phylogeny of the Clusiaceae using *rbc*L analyses.

Among the Central American species of *Clusia,* three morphological groups have been recognized (Hammel, 1986): the *Clusia flava* group, the *Clusia minor* group and the *Clusia multiflora* group. The three main clades of *Clusia* shown in our ITS analyses roughly correspond to these morphological groupings (Hammel, 1986), as the species of group I, II and III, by and large, match those of the previously established *C. flava, C. minor* and *C. multiflora* groups. There are few exceptions. These include *C. osseocarpa,* which has been categorized as a member of the *C. flava* group, yet in our analysis forms its own, although weakly supported branch between group I *(C. flava)* and II *(C. minor).* Morphological considerations have placed *C. rosea* and *C. valerioi* into the *C. minor* group (corresponding to group II); ITS sequencing moves these two species into group I (corresponding to *C. flava* group). Finally, *C. cupulata,* belonging to the *C. minor* group morphologically, appears in group III (the *C. multiflora* group) in both the NJ and the MP tree. Such exceptions to these morphological divisions are to be expected; the South American ssp. of *Clusia* display much more floral diversity than most of those of Central America, to which the morphological subdivisions pertain. *Clusia osseocarpa, C. rosea* + *C. valerioi,* and *C. cupulata* most likely belong to at least three distinct clades and are much more diverse in South America.

CAM

Our study suggests that CAM has evolved in only two of the three major groups of *Clusia* (Fig. **3**). Panamanian species known to show CAM occur in both the *C. flava* group (I) (i.e., *C. rosea, C. valerioi, C.* sp. D) and in the *C. minor* group (II) (i.e., *C. minor, C. uvitana, C.* sp. E). *C. minor, C. uvitana,* and *C.* sp. D are part of three highly supported clades. Given that the genus *Clusia* is monophyletic and assuming that CAM has evolved from a C_3 ancestor, our data support multiple, independent origins of CAM in the genus *Clusia.* Our data do not allow conclusions about possible reversals from CAM to C_3. Consistent with the position of Panamanian CAM species on the phylogenetic tree, most non-Panamanian *Clusia* species for which ITS sequence data are available (Vaasen et al., 2002) and which are known to exhibit strong CAM *(C. hilariana, C. fluminensis)* or weak CAM (e.g., *C. articulata, C. cruiva, C. parviflora*) also occur in group I when included in our phylogenetic calculations, and one species occurs in group II (Fig. **4**). A Panamanian species that has been shown to exhibit CAM under drought stress but was not included in our ITS analysis is *Havetiopsis flexilis* Spruce ex Planch. et TR (Zotz et al., 1999) that is synonymous to *Clusia flavida.* This species can be considered a member of the *C. flava* group (i.e., of group I) (B. E. Hammel, unpublished data). Interestingly, the very first report of CAM in a species of *Clusia* was with *C. lundelli* Standl. (Tinoco Ojanguren and Vásquez-Yanes, 1983), another member of the *C. flava* group (Hammel, 1986).

In contrast to group I and II, all the species of the *C. multiflora* group (III) show C_3 type $\delta^{13}C$ values and in none of these species has CAM been detected thus far using physiological performance criteria. Evidence based on CO_2 exchange studies of well-watered and water deficit-treated plants of *C. multiflora* (Grams et al., 1998) and *C.* sp. A (K. Winter, unpublished data) suggests that these two species are constitutive C_3 plants. Interestingly, in the NJ tree, the species of group III are characterized by relatively short, uniform branch length, as opposed to the species in groups I and II, which show longer, non-uniform branch lengths (Fig. **2**). Since branch length corresponds to the extent of sequence divergence, increased divergence in groups I and II would be consistent with photosynthetic pathway innovation (i.e., the development of CAM) that allows exploitation of an extended range of ecological opportunities.

The results shown here present a conservative view of the occurrence of CAM in Panamanian species of *Clusia.* $\delta^{13}C$ values alone do not reveal species in which CAM makes only a small contribution to total carbon gain (Winter and Holtum, 2002). When studying the origins of the CAM pathway in *Clusia,* species exhibiting weak CAM or occasional CAM (for example, as consequence of drought stress) must be treated with equal weight as species with strong CAM. A low degree of CAM can only be identified by rather time-consuming diel measurements of organic acid content and net CO_2 exchange of photosynthesizing tissues under a wide range of environmental conditions. With few exceptions, such measurements are missing for most Panamanian species of *Clusia* with C_3 type $\delta^{13}C$ values. These measurements, as well as DNA chip assays designed to assess subtle changes in gene expression of key CAM enzymes diagnostic of the degree of CAM use, are now a research priority to further evaluate the evolutionary origins of CAM in this remarkable group of tropical, arborescent plants.

Acknowledgements

This research was supported by grants from the Andrew W. Mellon Foundation through the Smithsonian Tropical Research Institute to J. C. C. and K. W., and by funds from the Smithsonian Tropical Research Institute to K. W. We thank Christopher Dick, Elisabeth King, Donald Windsor and Gwen Keller for comments on a previous version of the manuscript. J. C. C. acknowledges support of the University of Nevada Agricultural Experiment Station (NAES publication #0302515).

References

Baldwin, B. G., Sanderson, U. J., Porter, J. M., Wojciechowski, M. F., Campbell, C. S., and Donoghue, M. J. (1995) The ITS region of nuclear ribosomal DNA: a valuable source of evidence on Angiosperm phylogeny. Annals of the Missouri Botanical Garden 82, 247 – 277.

Borland, A. M. and Griffiths, H. (1996) Variations in the phases of crassulacean acid metabolism and regulation of carboxylation patterns determined by carbon-isotope-discrimination techniques. In Crassulacean acid metabolism. Biochemistry, ecophysiology and evolution. Ecological Studies, Vol. 114 (Winter, K. and Smith, J. A. C., eds.), Berlin, Heidelberg: Springer Verlag, pp. 230 – 249.

Crayn, D. M., Smith, J. A. C., and Winter, K. (2001) Carbon-isotope ratios and photosynthetic pathways in the neotropical family Rapateaceae. Plant Biology 3, 569 – 576.

D'Arcy, W. G. (1980) Flora of Panama. Guttiferae. Annals of the Missouri Botanical Garden 67, 969 – 1043.

Diaz, M., Haag-Kerwer, A., Wingfield, R., Ball, E., Olivares, E., Grams, T. E. E., Ziegler, H., and Lüttge, U. (1996) Relationships between carbon and hydrogen isotope ratios and nitrogen levels in leaves of *Clusia* species and two other Clusiaceae genera at various sites and different altitudes in Venezuela. Trees 10, 351 – 358.

Felsenstein, J. (1993) PHYLIP (Phylogeny inference package) Windows-version 3.5 c. Seattle: Universtiy of Washington.

Franco, A. C., Olivares, E., Ball, E., Lüttge, U., and Haag-Kerwer, A. (1994) *In situ* studies of crassulacean acid metabolism in several sympatric species of tropical trees of the genus *Clusia.* New Phytologist 126, 203 – 211.

Gehrig, H., Gaußmann, O., Marx, H., Schwarzott, D., and Kluge, M. (2001) Molecular phylogeny of the genus *Kalanchoe* (Crassulaceae) inferred from nucleotide sequences of the ITS-1 and ITS-2 regions. Plant Science 160, 827 – 835.

Grams, T. E. E., Herzog, B., and Lüttge, U. (1998) Are there species in the genus *Clusia* with obligate C_3 photosynthesis? Journal of Plant Physiology 152, 1 – 9.

Grams, T. E. E. and Thiel, S. (2002) High light-induced switch from C_3-photosynthesis to crassulacean acid metabolism is mediated by UV-A/blue light. Journal of Experimental Botany 53, 1475 – 1483.

Gustafsson, M. H. G., Bittrich, V., and Stevens, P. F. (2002) Phylogeny of Clusiaceae based on *rbc*L sequences. International Journal of Plant Science 163, 1045 – 1054.

Hammel, B. E. (1986) New species of Clusiaceae from Central America with notes on *Clusia* and synonymy in the tribe *Clusieae.* Selbyana 9, 112 – 120.

Holtum, J. A. M. and Winter, K. (1999) Degrees of crassulacean acid metabolism in tropical epiphytic and lithophytic ferns. Australian Journal of Plant Physiology 26, 749 – 757.

Jobes, D. V. and Thien, L. B. (1997) A conserved motif in the 5.8S ribosomal RNA (rRNA) gene is a useful diagnostic marker for plant internal transcribed spacer (ITS) sequences. Plant Molecular Biology Reporter 15, 326 – 334.

Jukes, T. H. and Cantor, C. R. (1969) Evolution of protein molecules. In Mammalian protein metabolism (Munro, H. N., ed.), New York: Academic Press, pp. 21 – 132.

Kimura, M. (1980) A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. Journal of Molecular Evolution 16, 111 – 120.

Liu, J. S. and Schardl, C. L. (1994) A conserved sequence in internal transcribed spacer 1 of plant nuclear rRNA genes. Plant Molecular Biology 26, 775 – 778.

Lüttge, U. (1991) *Clusia:* Morphogenetische, physiologische und biochemische Strategien von Baumwürgern im tropischen Wald. Naturwissenschaften 78, 49 – 58.

Lüttge, U. (1996) *Clusia:* plasticity and diversity in a genus of C_3/CAM intermediate tropical trees. In Crassulacean acid metabolism. Biochemistry, ecophysiology and evolution. Ecological Studies 114 (Winter, K. and Smith, J. A. C., eds.), Berlin, Heidelberg: Springer-Verlag, pp. 296 – 311.

Lüttge, U. (1999) One morphotype, three physiotypes: sympatric species of *Clusia* with obligate C_3 photosynthesis, obligate CAM and C_3-CAM intermediate behaviour. Plant Biology 1, 138 – 148.

Maguire, B. (1979) On the genus *Clusia* (Clusiaceae) in Mexico. Taxon 28, 13 – 18.

Mayol, U. and Rossello, J. A. (2001) Why nuclear ribosomal DNA spacers (ITS) tell different stories in *Quercus.* Molecular Phylogenetics and Evolution 19, 167 – 176.

Pierce, S., Winter, K., and Griffiths, H. (2002) Carbon isotope ratio and the extent of daily CAM use by Bromeliaceae. New Phytologist 156, 75 – 84.

Pipoly, J. J. I. and Graff, A. (1995) A synopsis of the genus *Clusia* sections *Criuvopsis* and *Brachystemon* (Clusiaceae) in northern South America. Sida 16, 505 – 528.

Pipoly, J. J., Kearns, D. M., and Berry, P. E. (1998) *Clusia.* In Flora of the Venezuelan Guayana, Vol. 4, Caesalpiniaceae-Ericaceae (Berry, P. E., Holst, B. K., and Yatskievych, K., eds.), St. Louis, Missouri: Missouri Botanical Garden Press, pp. 260 – 294.

Sambrook, J. and Russell, D. W. (2001) Molecular cloning: a laboratory manual. New York: Cold Spring Harbor Laboratory Press.

Swofford, D. L. (2002) PAUP*, version 4.0 b8 a. Sunderland, Massachusetts: Sinauer Associates.

Ting, I. P., Lord, E. M., Sternberg da, S. L., and DeNiro, M. J. (1985) Crassulacean acid metabolism in the strangler *Clusia rosea* Jacq. Science 229, 969 – 971.

Tinoco Ojanguren, C. and Vásquez-Yanez, V. (1983) Especies CAM en la selva húmeda tropical de los Tuxtlas, Veracruz. Boletín de la Sociedad Botánica de México 45, 150 – 153.

Vaasen, A., Begerow, D., Lüttge, U., and Hampp, R. (2002) The genus *Clusia* L.: molecular evidence for independent evolution of photosynthetic flexibility. Plant Biology 4, 86 – 93.

Valiejo-Roman, C. M., Terentiera, E. I., Samigullin, T. H., and Pimener, M. G. (2002) Relationships among genera in Saniculoideae and selected Apioideae (Umbelliferae) inferred from nrITS sequences. Taxon 51, 91 – 101.

Wanek, W., Huber, W., Arndt, S. K., and Popp, M. (2002) Mode of photosynthesis during different life stages of hemiepiphytic *Clusia* species. Functional Plant Biology 29, 725 – 732.

White, T. J., Bruns, T., Lee, S., and Taylor, J. (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In PCR protocols: a guide to methods and applications (Innis, M. A., Gelfand, D. H., Sninsky, J. J., and White, T. J., eds.), San Diego: Academic Press, pp. 315 – 322.

Winter, K. and Holtum, J. A. M. (2002) How closely do the $\delta^{13}C$ values of crassulacean acid metabolism plants reflect the proportion of CO_2 fixed during day and night? Plant Physiology 129, 1843 – 1851.

Winter, K. and Smith, J. A. M. (eds.) (1996) Crassulacean acid metabolism. Biochemistry, ecophysiology and evolution. Ecological Studies, Vol. 114. Berlin, Heidelberg: Springer-Verlag.

Winter, K., Zotz, G., Baur, B., and Dietz, K.-J. (1992) Light and dark CO_2 fixation in *Clusia uvitana* and the effects of plant water status and CO_2 availability. Oecologia 91, 47 – 51.

Yuan, Y. M. and Küpfer, P. (1995) Molecular phylogenetics of the subtribe Gentianinae (Gentianaceae) inferred from the sequences of internal transcribed spacers (ITS) of nuclear ribosomal DNA. Plant Systematics and Evolution 196, 207 – 226.

Zotz, G. and Winter, K. (1993) Short-term regulation of crassulacean acid metabolism activity in a tropical hemiepiphyte, *Clusia uvitana.* Plant Physiology 102, 835 – 841.

Zotz, G. and Winter, K. (1994 a) Annual carbon balance and nitrogen-use efficiency in tropical C_3 and CAM epiphytes. New Phytologist 126, 481 – 492.

Zotz, G. and Winter, K. (1994 b) A one-year study on carbon, water and nutrient relationships in a tropical C_3- and CAM hemiepiphyte, *Clusia uvitana* Pittier. New Phytologist 127, 45 – 60.

Zotz, G., Reichling, P., and Krack, S. (1999) Another woody hemiepiphyte with CAM: *Havetiopsis flexilis* SPRUCE ex PLANCH. Et TR. (Clusiaceae). Flora 194, 215 – 220.

K. Winter

Smithsonian Tropical Research Institute
P.O. Box 2072
Balboa, Ancon
Republic of Panama

E-mail: winterk@tivoli.si.edu

Section Editor: H. Rennenberg