The Genus *Clusia* L.: Molecular Evidence for Independent Evolution of Photosynthetic Flexibility

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Abstract: **Members of the Clusiaceae genus *Clusia* (tropical trees and shrubs) belong to the small group of dicotyledonous trees which are able to perform crassulacean acid metabolism (CAM). Most of the species are able to switch between C_3 and CAM modes of photosynthesis and only a few are restricted to either C_3 or CAM. In order to discover possible phylogenetic relationships with regard to the mode of photosynthesis, we investigated 17 species of the genus *Clusia,* and one species each of the Clusiaceae genera *Oedematopus* and *Hypericum* on the basis of internal transcribed spacer (ITS) sequences between the 18S and 26S coding regions of nuclear ribosomal DNA. Little length variation was detected in the ITS region of *Clusia* species. ITS1 sequences ranged from 255 to 260 bp and ITS2 sequences from 208 to 210 bp. Neighbour-joining and parsimony analyses of these sequences resulted in considerable differences in cluster formation when compared to a classification based on morphological characteristics. The molecular data also give no indication of a group-specific evolution of modes of photosynthesis, i.e., C_3 and CAM. We thus conclude that CAM has evolved independently several times within the genus *Clusia.***

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

Abbreviations:

CAM:	crassulacean acid metabolism
EDTA:	ethylenediamine tetraacetic acid
ITS:	internal transcribed spacer
PAUP*:	phylogenetic analysis using parsimony (*and other methods)
PCR:	polymerase chain reaction
TAE:	tris-glacial acetic acid-EDTA

Introduction

Clusia L. (Fam. Clusiaceae/Hypericaceae/Guttiferae, Order Theales) is a highly diverse and physiologically flexible genus of tropical shrubs and trees. It contains 145 (Willis, 1973[55]) to 300 species (Pipoly et al., 1998[41]), which occur in South America, Madagascar and New Caledonia in a wide range of different life forms and habitats, such as coastal sand dunes, borders of savannas or upper montane cloud forests (Lüttge, 1991[29]). All species possess entire, leathery leaves. Two species, *Clusia rosea* Jacq. and *C. minor* L., are widely cultivated throughout the tropics (Pipoly and Graff, 1995[40]).

These trees and shrubs belong to the few dicotyledonous trees performing crassulacean acid metabolism (CAM), which is an ecophysiological adaptation of plants to arid environments (Winter and Smith, 1996[56]). They show an extraordinary plasticity in the expression of CAM in response to changes in environmental factors, such as light, temperature and water availability (Borland et al., 1992[9]; Zotz and Winter, 1993[60]). Most of the species studied so far have the capacity to perform CAM, being either obligate CAM species or C_3/CAM intermediates, and only a few are presumed to be obligate performers of C_3 photosynthesis (Grams et al., 1997[16]; Herzog et al., 1999[23]).

CAM plants reduce transpiratory water loss by taking up CO_2 during the night when stomates are open and the leaf to air water vapour pressure deficit is low. They fix CO_2 via the enzyme phospho*enol*pyruvate carboxylase (PEPC) and store it overnight in the vacuole as malic acid. During the day, stomata are closed and CO_2 resulting from the decarboxylation of malate is used by the Calvin cycle enzyme, ribulose bisphosphate carboxylase/oxygenase (Rubisco) (Grams et al., 1998[17]). Under sufficient water supply, these plants also show gas exchange during the day. The additional carbon gain via Rubisco is the basis for growth. Within the genus *Clusia,* some species show an enormous flexibility in switching from the (most common) C_3 metabolism of photosynthesis to CAM, and back, in response to changes in the environment (Ball et al., 1991[6]; Borland et al., 1992[9]; Franco et al., 1992[14]; Haag-Kerwer et al., 1996[19]). Two species using different photosynthetic pathways even co-exist in the same savanna habitat in Venezuela (Franco et al., 1994[15]). Only a few of the species of the genus studied so far appear to be restricted to either the C_3 type (*Clusia multiflora* H. B. K.; Grams et al., 1998[17]; Herzog et al., 1999[24]) or CAM type of photosynthesis (*Clusia alata* Planch. et Triana and *C. hilariana* Schlecht.; Herzog et al., 1999[23]).

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Table 1 Plant material, sources of the plant material (Darmstadt = Botanical Garden of the Technical University of Darmstadt; Bochum = Botanical Garden of the Ruhr University Bochum; Tübingen = Botanical Garden of the Eberhard-Karls University Tübingen), length of the entire ITS region containing ITS1, 5.8S and ITS2 region of 17 *Clusia* species, one *Oedematopus* species, and one *Hypericum* species as outgroup, and accession numbers of the DNA sequences in the EMBL Sequence Data Library

Plant material	Source	ITS length	Accession number
Clusia alata Planch. et Triana	Darmstadt	622 bp	AJ414710
C. aripoensis Britton	Darmstadt	623 bp	AJ414711
C. articulata Vesque	Darmstadt	622 bp	AJ414712
C. criuva Vesque	Darmstadt	622 bp	AJ414713
C. fluminensis Planch. et Triana	Darmstadt	623 bp	AJ414714
C. hilariana Schlecht.	Darmstadt	622 bp	AJ414715
C. lanceolata Cambess.	Darmstadt	621 bp	AJ414716
C. major L.	Darmstadt	621 bp	AJ414717
C. minor L.	Darmstadt	622 bp	AJ414718
C. multiflora H. B. K.	Darmstadt	620 bp	AJ414719
C. nemorosa G. F. W. Mey.	Darmstadt	620 bp	AJ414720
C. parviflora Engl.	Darmstadt	621 bp	AJ414721
C. rosea Jacq.	Darmstadt	622 bp	AJ414722
C. spec. L.	Darmstadt	622 bp	AJ414723
C. tocuchensis Britton	Darmstadt	624 bp	AJ414724
C. uvitana Pittier	Bochum	620 bp	AJ414725
C. venosa Jacq.	Darmstadt	622 bp	AJ414726
Oedematopus obovatus Planch. et Triana	Darmstadt	620 bp	AJ414727
Hypericum calycinum L.	Tübingen	633 bp	AJ414728

Studies on molecular phylogenetics have demonstrated that the internal transcribed spacer (ITS) region of ribosomal DNA (rDNA), defined as the unit containing the ITS1 spacer, 5.8S rRNA gene, and ITS2 spacer, is very useful to reconstruct phylogenies at various taxonomic levels (Baldwin, 1993[4]; Baldwin et al., 1995[5]; Hershkovitz and Lewis, 1996[21]; Hershkovitz and Zimmer, 1996[22]).

ITS1 and ITS2 regions have been used successfully in many recent phylogenetic studies of angiosperms at lower taxonomic levels, such as among genera or species (Baldwin, 1992[3]; Baldwin et al., 1995[5]). The sequences have very little variation in length at the generic level in flowering plants and are easy to align. They are of sufficient length for phylogenetic reconstructions, and they are flanked by regions that are highly conserved within genera because, in general, the sequences of spacer regions evolve more rapidly than coding regions (Suh et al., 1993[50]). In addition, the relatively small size (usually 600 – 700 bp) of the ITS region and the special locations of the spacers, being flanked by highly conservative coding regions, are very favourable for primer design and direct sequencing of polymerase chain reaction (PCR) amplified products (Yuan and Küpfer, 1995[59]). Sequence motifs conserved throughout angiosperms have been recognized (Liu and Schardl, 1994[28]), and the 5.8S rDNA region retained a sufficient phylogenetical signal to distinguish among sequences from green algae, fungi and seed plants (Jobes and Thien, 1997[25]).

In this study we have analysed ITS sequences to construct phylogenetic relationships of the genus *Clusia.* Based on 17 different species, we show that there is no obvious correlation with the mode of photosynthesis.

Materials and Methods

We analysed ITS sequences of 17 species of the genus *Clusia,* one of the genus *Oedematopus,* and one of the genus *Hypericum. Oedematopus* is closely related to *Clusia* and now incorporated in the genus *Clusia* as a subsection (Pipoly et al., 1998[41]). *Hypericum* is in the same familiy, in some floras taken as Clusiaceae in others as Hypericaceae, but it is clearly a different genus. *Oedematopus* has facultative CAM capacity (Lüttge, 2000[31]) while, on the other hand, there are no known CAM species in the genus *Hypericum.*

Plants of *Clusia* and *Oedematopus* were from the live collection of the Botanical Garden, Department of Biology, Technical University of Darmstadt, where plants have been grown for many years in glasshouses in pot culture. *Clusia uvitana* Pittier was obtained from the Botanical Garden of Ruhr University, Bochum and *Hypericum calycinum* was from the Botanical Garden of Eberhard-Karls University, Tübingen (Table **1**).

DNA extraction

Fresh leaves were cut in pieces of 150 to 250 mg, frozen in liquid nitrogen and stored at – 80 °C until use. For the extraction of total genomic DNA (DNeasy® Plant Mini Kit; Qiagen GmbH, Hilden, Germany), 150 to 250 mg of leaf tissue were homogenized in liquid nitrogen.

PCR reaction

The entire ITS region, comprising ITS1, the 5.8S gene and ITS2, was amplified by PCR using the external "ITS1" and "ITS4" primers designed by White et al. (1990[54]).

PCRs were carried out in 50 µl reaction mixtures containing 5 µl of 10 × Expand High Fidelity buffer plus 15 mM $MgCl_2$ (Roche Molecular Biochemicals, Mannheim, Germany), 1 µl of dNTPs (Taq Core Kit, Qiagen), 1 µl each of primers ITS1 and ITS4 (30 pmol), 2.5 units of the enzyme mix of Taq DNA polymerase plus proofreading polymerase (Roche Molecular Biochemicals), 40.3 µl of sterile double-distilled water and 1 µl of genomic DNA (~ 100 pg).

PCR was performed using a Peltier Thermal Cycler (Biozym Diagnostics, Hess. Oldendorf, Germany) under the following conditions: initial denaturation at 94 °C for 5 min, followed by 35 cycles of template denaturation at 94 °C for 30 sec, primer annealing at 50 °C for 1 min, primer extension at 72 °C for 1 min and a final extension at 72 °C for 5 min.

Purification of PCR products

Five µl of each PCR reaction were separated on a 1 % agarose gel using 0.5 × TAE as the gel buffer, to check for the quality of amplification. Successful PCR amplifications resulted in a single DNA band corresponding to ~ 700 bp. ITS fragments were purified with the MinElute PCR Purification Kit (Qiagen).

Purified fragments (3.5 µl) were ligated into pCR®2.1-TOPO® vector or pCR®II-TOPO® vector (Invitrogen BV, Groningen, Netherlands) and used to transform TOP 10 cells (Invitrogen). Plasmid DNA was isolated according to Sambrook and Russel (2001[46]), purified (QIAprep Spin Miniprep Kit, Qiagen) and sequenced (LI-COR DNA Sequencer 4200, Sequ-Therm EXCEL II-DNA Sequencing Kit-LC, Biozym Diagnostics). Sequencing was done at the University of Würzburg (Germany, Prof. R. Kaldenhoff). The number of sequenced plasmids with ITS inserts per genus was one to three.

Sequence alignment and phylogenetic analyses

The boundaries of ITS1, 5.8S and ITS2 were determined by comparison with various published sequences available in GenBank at NCBI (National Centre for Biotechnology Information). Sequences were aligned using the ClustalW algorithm of Megalign (Lasergene; DNASTAR Inc., Madison, Wisconsin) and were visually optimized.

Phylogenetic analyses were performed using PAUP, version 4.0b8 (Swofford, 1999[52]). Modeltest 3.06 (Posada, 1998[43]) was used to select the optimal substitution model. Neighbour joining was calculated with TrNef+G (parameters: equal base frequencies; substitution matrix = [1.0000 5.1917 1.0000 1.0000 10.1813]; gamma distribution with a = 0.2308) and GTR + G (base frequencies = [0.2313 0.2812 0.2727]; substitution matrix = [0.4922 3.3346 0.8914 0.3428 6.7538]; gamma distribution with a = 0.2382) as substitution models. The test for maximum parsimony was carried out in a two step analysis: in a first step we searched for different islands using 10 000 random additions without swapping, then we optimized the trees obtained with an extended swapping procedure (TBR). Bootstrapping was performed with 1000 replicates.

Results

Characteristics in the ITS region

Length variation for the entire ITS region, including ITS1, 5.8S rDNA and ITS2 is between 619 and 631 bp (Table **1**). The ITS1 region (255 to 260 bp) is longer than ITS2 (208 to 210 bp). The outgroup species *Hypericum calycinum* (L.) exceeds these ranges, exhibiting an ITS1 length of 244 bp and an ITS2 length of 231 bp. The 5.8S DNA consists of 156 bp (sites 278 to 433) and is highly conserved in all species, and only eleven 1 bp variants could be detected. We also found the conserved angiosperm motif (5′-GAATTGCAGAATCC) within the 5.8S rRNA gene (sites 351 to 364), which can be used to differentiate between flowering plants, fungi and algae (Jobes and Thien, 1997[25]). Gaps occur only twice in the sequence of *Oedematopus obovatus* (Planch. et Triana), at sites 409 and 410. At the beginning and at the end of our amplified product there are also conserved regions belonging to the 18S rDNA (sites 1 to 11) and to the 26S rDNA (sites 667 to 704).

The ITS1 region varies between 244 and 260 bp (sites 12 to 277). Successful sequence alignments of ITS1 required 20 gaps of 1, 4 or 8 bp occurring in the outgroup taxa *H. calycinum* and 6 to 11 gaps of 1 or 2 bp occurring in individual sequences of the ingroup species. ITS2 regions vary from 208 to 231 bp (sites 434 to 666). Alignments of ITS2 required 23 to 25 gaps of 1 to 6 bp occurring in individual sequences of the ingroup taxa, while the outgroup species has only 2 gaps at sites 629 and 630.

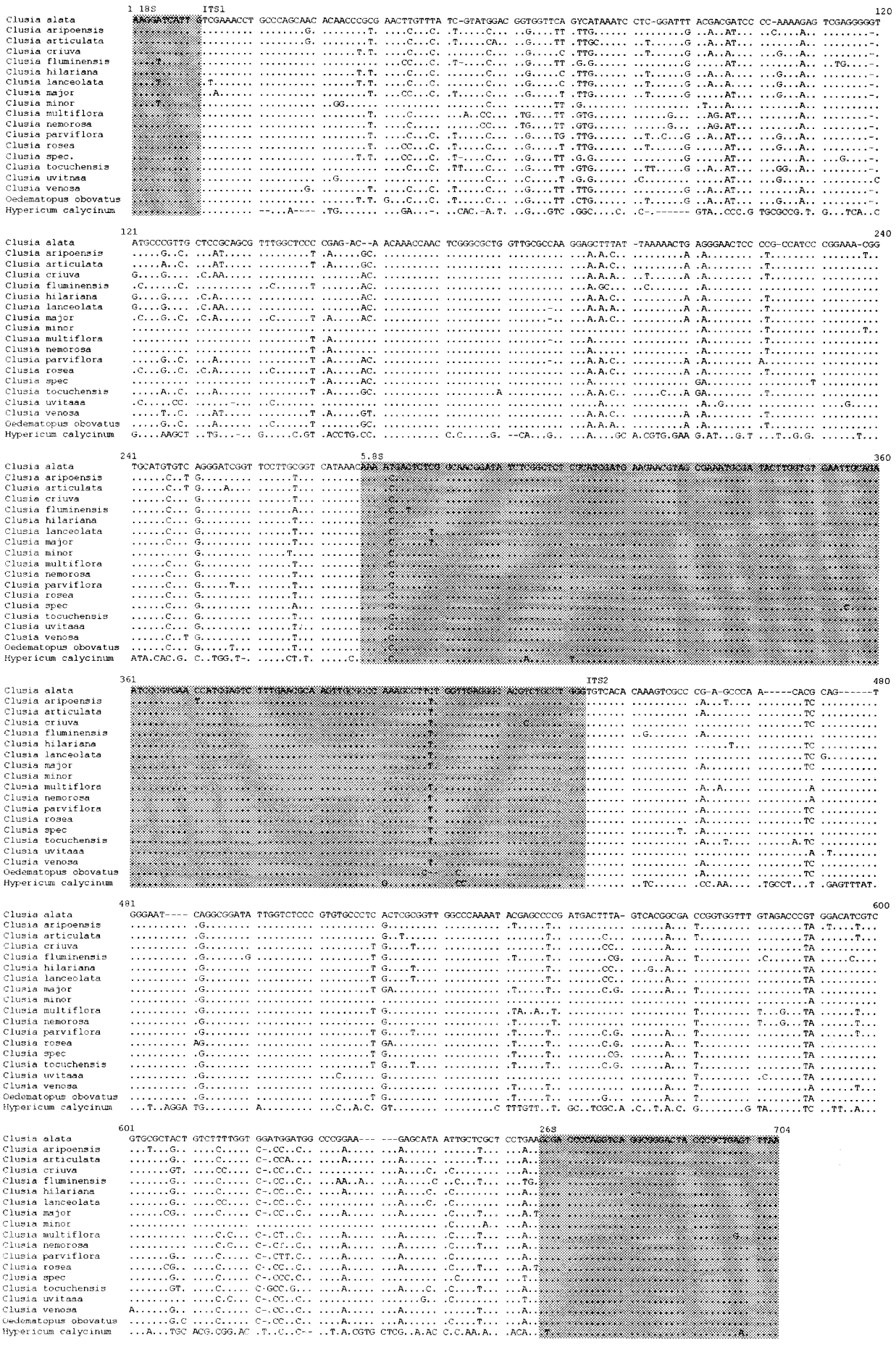
Out of the 655 aligned positions in the entire fragment length, 61 sites involve gaps (32 in ITS1, 2 in 5.8S rRNA and 27 in ITS2); 368 sites (112 in ITS1, 145 in 5.8S rRNA and 111 in ITS2) are identical for all the sequences surveyed; at 130 sites (72 in ITS1, 2 in 5.8S rRNA and 56 in ITS2) the outgroup species is different from the other taxa; 35 sites (16 in ITS1, 6 in 5.8S rRNA and 13 in ITS2) show nucleotide divergence at only one ingroup species; and 122 sites (66 in ITS1, 3 in 5.8S rRNA and 53 in ITS2) present differences of more than one nucleotide (Fig. **1**).

Phylogenetic analysis of ITS sequences

The Modeltest analysis results in two different substitution models for the likelihood ratio test and the Akaike Information Criterion. Neighbour joining analysis with each of these two models results in more or less the same topology. In Fig. **2** we present the results of the neighbour joining analysis with GTR+G as substitution model. Maximum parsimony resulted in eight most parsimonious trees (Fig. **3**).

Fig. 1 Aligned sequences of the ITS region from 17 *Clusia* species, *Oedematopus obovatus,* and of *Hypericum calycinum* as outgroup. Columns are nucleotide sites, and rows are individual DNA sequences. Nucleotide sequence is displayed from 5′ to 3′. Gaps are indicated by "–", and the coding regions for the ribosomal DNA by a grey background. ITS1 ranges from site 12 to 277, 5.8S coding region from site 278 to 433 and ITS2 from site 434 to 666. Sites 1 to 11 belong to the end of the 18S coding region and sites 667 to 704 identify the beginning of the 26S coding region. ▶

Fig. 1


1 18S ITS1 120
Clusia alata
Clusia aripoensis
Clusia articulata
Clusia criuva
Clusia fluminensis
Clusia hilariana
Clusia lanceolata
Clusia major
Clusia minor
Clusia multiflora
Clusia nemorosa
Clusia parviflora
Clusia rosea
Clusia spec.
Clusia tocuchensis
Clusia uvitnaa
Clusia venosa
Oedematopus obovatus
Hypericum calycinum
121 240
241 5.8S 360
361 ITS2 480
481 600
601 26S 704

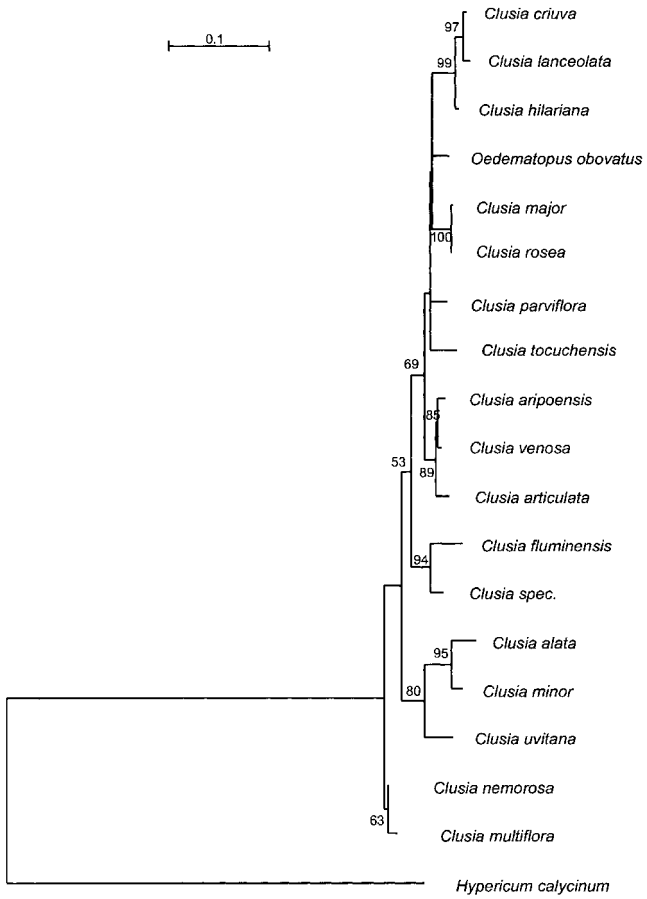


Fig. 2 Phylogenetic hypothesis derived from neighbour joining analysis of a 704 bp alignment of ITS sequences of the nuclear encoded rDNA gene, rooted with *Hypericum calycinum*. The general time-reversible model with gamma distribution was used as substitution model. Bootstrap values (1000 replicates) below 50% are not shown. Bar = 0.02 expected changes per site.

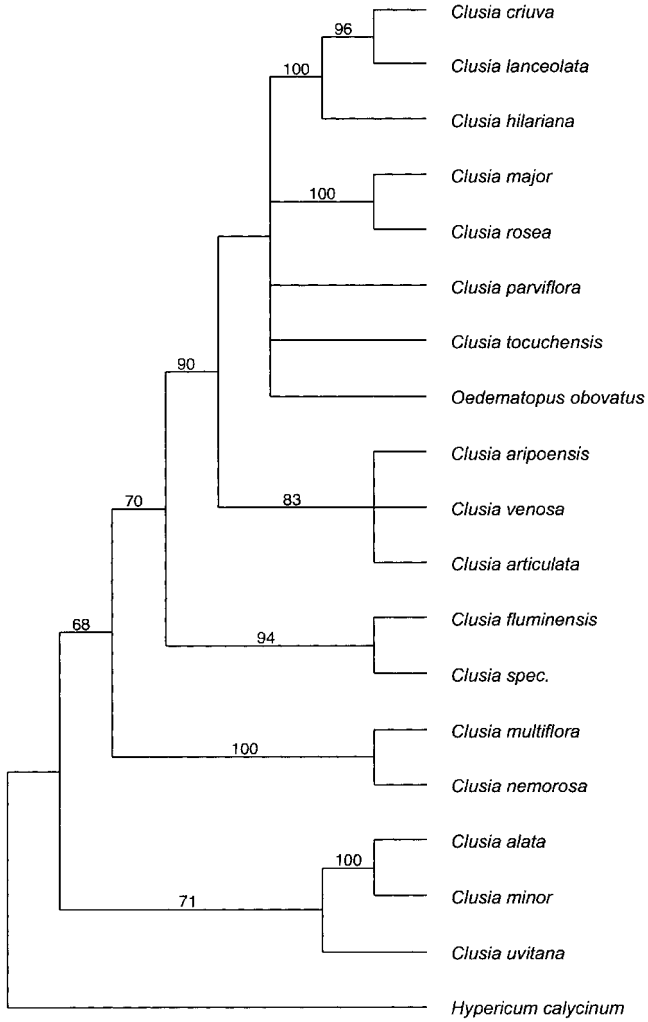


Fig. 3 Majority rule consensus tree of 8 most parsimonious trees (402 steps) found by heuristic analysis of a 704 bp alignment of ITS sequences of the nuclear encoded rDNA gene, rooted with *Hypericum calycinum*. Bootstrap values (1000 replicates) below 50% are not shown.

The different types of analysis result, however, in very similar topologies. The sequence of *H. calycinum* was arranged in different positions with a very long distance. This might cause a long branch attraction problem and may explain the differences in the varying reconstruction methods. In contrast, the main groups are obvious in all topologies. The terminal clusters consisting of *Clusia alata, C. minor* and *C. uvitana* or *C. hilariana, C. lanceolata* (Cambess.) and *C. criuva* (Vesque) are supported by all analyses (Figs. **2, 3**). Analysis of the ITS sequence data group very often locates *Oedematopus obovatus* within the genus *Clusia*. In simpler models we obtain only once a position basal to *Clusia,* but without any bootstrap support.

The trees derived clearly show that there are some monophyletic groups. *Clusia criuva, C. lanceolata* and *C. hilariana* constitute one group and *C. rosea* and *C. major* (L.) another. The third group we could identify includes *C. alata, C. minor* and *C. uvitana* and a fourth group is formed by *C. nemorosa* (G. F. W. Mey.) and *C. multiflora*. Interestingly, we obtain high bootstrap support for a larger subgroup formed by *C. criuva, C. lanceolata, C. hilariana, O. obovatus, C. major, C. rosea, C. parviflora* (Engl.), *C. tocuchensis* (Britton), *C. aripoensis* (Britton), *C. venosa* (Jacq.), and *C. articulata* (Vesque) in all analyses (Figs. **2, 3**).

Discussion

General characteristics of the ITS sequences of the species investigated

All DNA fragments sequenced so far contain a conserved motif in the ITS1 region which is typical for angiosperms (GGCRY-[4 to 7 n]-GYGYCAAGGAA; Liu and Schardl, 1994[28]). In the studied species, this motif is located at positions 174 through 194, and the consensus sequence is GGCGC-TGGTT-GCGCCAAGGAA. In contrast, we could not identify a corresponding motif in the ITS 2 region (see Liu and Schardl, 1994[28]). This region is obviously less conserved.

Within the ingroup, the length of ITS1 is 255 to 260 bp, that of ITS2 208 to 210 bp. In contrast, the corresponding sequences in the outgroup contain 244 and 231 bp.

ITS1 and ITS2 fragment lengths can be equal or different. For all *Clusia* spec. investigated so far, we found that ITS1 sequences were shorter than ITS2. This is similar to a range of other angiosperm families, such as Asteraceae (Baldwin, 1992[3]; Baldwin, 1993[4]; Bayer et al., 1996[7]; Kim and Jansen, 1994[27]; Sang et al., 1994[47]; Susanna et al., 1995[51]), Brassicaceae (Francisco-Ortega et al., 1999[13]; Yang et al., 1999[58]), Fabaceae (Ainouche and Bayer, 1999[1]; Käss and Wink, 1997[26]; Wojciechowski et al., 1993[57]), Gesneriaceae (Möller and Cronk, 1997[38]), Paeoniaceae (Sang et al., 1995[48]), Rosaceae (Alice and Campbell, 1999[2]) and Winteraceae (Suh et al., 1993[50]).

Phylogenetic relationships between Clusia *species and their mode of photosynthesis*

All phylogenetic analyses of our sequence data led to nearly identical grouping of species. We thus conclude that the ITS sequences obtained from *Clusia* are useful for a phylogenetic interpretation of this genus.

The trees derived clearly show that *C. rosea* and *C. major* constitute a monophyletic group. D'Arcy (1980[10]) called them synonymous, and also on the basis of our results it is quite reasonable to assume that these two species are identical.

Another monophyletic group we could identify consists of *C. criuva, C. lanceolata* and *C. hilariana.* This arrangement is supported in several ways. Two of these species, *C. lanceolata* and *C. hilariana,* have been combined in the subsection *Phloianthera* (Engler, 1858 – 1879[12]; Planchon and Triana, 1860[42]; Vesque, 1893[53]), which is also consistent with data on the composition of floral fragrances (Nogueira et al., 2001[39]). In a first attempt to establish phylogenetic relationships (Lüttge, 2000[31]), *C. criuva* and *C. lanceolata* also formed a subgroup. On the other hand, *C. criuva* is often separated in an extra subsection, namely *Criuva* (Nogueira et al., 2001[39]; Planchon and Triana, 1860[42]; Pipoly et al., 1998[41]). In the Flora Brasiliensis, *C. criuva* and *C. parviflora* are part of the subsection *Eucriuva* (Engler, 1858 – 1879[12]), and this is also supported by the data presented here, as well as by other recent data (Nogueira et al., 2001[39]).

C. parviflora and *C. minor* L. have been regarded as synonymous (D'Arcy, 1980[10]; Planchon and Triana, 1860[42]). Our data, however, clearly indicate that these two species are well distinguishable from each other, and thus most probably not identical.

Another monophyletic group includes *C. alata, C. minor* and *C. uvitana.* A fourth group is formed by *C. nemorosa* (G. F. W. Mey.) and *C. multiflora.*

Planchon and Triana (1860[42]), as well as Pipoly et al. (1998[41]) and Vesque (1893[53]) located *C. nemorosa* and *C. rosea* in the subsection *Euclusia,* while *C. multiflora* and *C. alata* were assigned to the subsection *Anandrogyne* (Planchon and Triana, 1860[42]; Vesque, 1893[53]). This is again in contrast to our sequence data, where the species are related in a completely different way.

Hammel (1986[20]) arranged *C. minor, C. uvitana* and *C. rosea* in one morphological group. In our approach, *C. minor* and *C. uvitana* are very closely related to each other, while *C. rosea* is well separated from these two species. *C. minor* is also regarded as synonymous with *C. venosa* and *C. uvitana* (D'Arcy, 1980[10]). However, according to our results, *C. venosa* is nearly as far away from *C. minor* as *C. parviflora,* while *C. alata* is more closely related to *C. minor* than *C. uvitana.* It is thus reasonable to assume that *C. minor, C. venosa* and *C. uvitana* are different species.

Accordingly, the subgroup with high bootstrap support, containing *C. criuva, C. lanceolata, C. hilariana, Oedematopus obovatus, C. major, C. rosea, C. parviflora, C. tocuchensis, C. aripoensis, C. venosa* and *C. articulata,* differs from the conventional classification. This finding is corroborated by another recent approach, making use of a RAPD analysis (Random Amplified Polymorphic DNA; Lüttge, 2000[31]). Major differences between both types of analysis are that, according to the RAPD data, *C. minor* and *C. multiflora* are also part of this group while they are not included on the basis of sequence data. Furthermore, RAPD analysis identifies *O. obovatus* as an outgroup, which is not the case in this study. The latter is also supported by floral morphology (Gustafsson, 2000[18]).

The comparison of our phylogenetic tree with traditional systematics of the genus *Clusia* is very difficult because of the rather limited range of data available. There are mainly site-specific descriptions of different *Clusia* species in the literature. The existing classifications mostly refer just to the country of origin (D'Arcy, 1980[10]; Engler, 1858 – 1879[12]; Hammel, 1986[20]; Maguire, 1958[32]; Maguire, 1961[33]; Maguire, 1966[34]; Maguire, 1978[36]; Maguire, 1979[37]; Pipoly and Graff, 1995[40]; Pipoly et al., 1998[41]; Standley, 1937[49]). Diaz et al. (1996[11]) tried to distinguish between the different species and their synonyms. This appeared to be extremely difficult. For example, Pipoly and Graff (1995[40]) described *Clusia* as a genus with 250 species, but 336 names, expecting 40 more undescribed species in the Andes of Colombia, Ecuador, Peru and Bolivia. They also regard the taxonomy of this group as difficult because of its sexual lability, with staminate, pistillate, bisexual (Hammel, 1986[20]) and apomictic individuals (Hammel, 1986[20]; Maguire, 1976[35]). Bittrich and Amaral (1996[8]) commented on the lack of knowledge about the phylogeny of *Clusia.* They reported that the species with hermaphrodite flowers belong to three different sections of the genus and that they are not monophyletic. Nogueira et al. (2001[39]) also concluded that the traditional sections of *Clusia,* based on morphological characteristics, are not monophyletic.

Just as with the obvious difficulties in correlating morphological characteristics with data obtained by molecular systematics, it is also not possible to find any hints of a group-specific evolution of CAM. Species performing CAM can be identified by their low carbon isotope discrimination. Values of $\delta^{13}C$ well above – 20‰ indicate prevailing primary carboxylation via PEPC, i.e., CAM as long as C_4 photosynthesis is excluded as is the case in *Clusia* (see Lüttge, 1999[30], 2000[31]). Such values were recorded for *C. rosea* (– 16.6‰), *C. major* (– 16.0‰), *C. hilariana* (– 17.2‰), *C. fluminensis* (– 16.6‰) and *C. alata* (– 15.3‰) (see Lüttge, 1999[30], 2000[31]). Apart from *C. rosea* and *C. major,* which may be the same species (see above), these strong CAM-performing species are statistically spread over all phylogenetic groups (Figs. **2, 3**). The other species have $\delta^{13}C$ values ranging from – 23 to – 30‰. Since there are many C_3/CAM intermediate species in the genus *Clusia,* which some-

times perform C_3 photosynthesis, and also obligate CAM species, which may make use of daytime phases of CO_2 fixation directly via Rubisco with a higher carbon isotope discrimination than PEPC, it is clear that species having CAM capacity are still part of this group, as shown by physiological and biochemical analyses (Lüttge, 1999[30]). However, most notably *C. multiflora,* which is thought to be a *bona fide* obligate C_3 species ($\delta^{13}C$ – 26.2‰; Grams et al., 1998[17]), is not located at the bottom of the tree, and thus seems not to be the origin of the CAM-performing or C_3/CAM-switching species. This supports our view that the ability to perform CAM has evolved several times in different phyletic groups of *Clusia.* Evolution of CAM is generally considered a recent phylogenetic event (Raven and Spicer, 1996[44]). Therefore, an alternative explanation that the whole genus has evolved CAM from other C_3 ancestors and that the CAM potential was lost secondarily in, e.g., *C. multiflora,* appears less likely. A comparison with the evolution of C_4 photosynthesis strengthens this interpretation: more than 30 distinct origins of this pathway have been identified, even multiple independent ones in one family (Sage, 2001[45]).

Based on the data available, we thus conclude that the ability for photosynthetic acclimation has evolved independently within the different subgroups of *Clusia.* Clearly, this conclusion has to be verified by an extended species-specific analysis of physiological properties and DNA sequence data.

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