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Evolution of spines and the taxonomic status of *Convolvulus* section *Acanthocladi*: preliminary results from the ITS 2 region of nrDNA

Abstract

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A molecular phylogenetic analysis using the Internal Transcribed Spacer 2 region (ITS 2) of nuclear ribosomal DNA was conducted to investigate the evolution of spines within *Convolvulus* and to test the monophyly of section *Acanthocladi*. A total of 49 species were included in the analysis, comprising 40 species of *Convolvulus*, *Calystegia sepium* and eight outgroup taxa. The results from parsimony analysis of the data showed that *C. caput-medusae*, a spined species from the Canary Islands, is more closely related to other Canarian endemic species of *Convolvulus* than to the spined *C. trabutianus*, from Morocco, with which it has been previously considered conspecific. Spines are shown to be homoplastic within *Convolvulus* and eight steps are required to optimise the character on the most parsimonious tree presented. Section *Acanthocladi* together with the other two sections recognised in the most recent revision of *Convolvulus* from Western Asia, the Mediterranean and Macaronesia are shown to be polyphyletic.

Introduction

Convolvulus L. (*Convolvulaceae*) comprises approximately 200 species worldwide. An important centre of diversity for the genus is found in Western Asia, the Mediterranean and Macaronesia and within this region a wide range of growth forms are represented. These include erect, prostrate and climbing herbs, compact cushion plants, shrubs and subshrubs (some of which show a tendency towards twining of their uppermost branches), robust lianas, and a number of spine-bearing shrubs and subshrubs, found only in the more arid parts of the region.

Boissier (1875), in his treatment of *Convolvulus* for *Flora Orientalis*, used the diversity in growth form, together with ovary pubescence, to delimit 10 infrageneric groups within the genus. Sa'ad (1967), in a later revision dealing with all species of *Convolvulus* from Western Asia, the Mediterranean and Macaronesia, modified Boissier's classification scheme and recognised three sections and 12 subsections within the genus. The three sections recognised by Sa'ad (1967) were characterised as follows:

Section *Acanthocladi*: shrubs or subshrubs possessing spines.

Section *Inermes*: shrubs and subshrubs with branches that are erect or prostrate but never twining.

Section *Convolvulus*: herbs and subshrubs with twining branches.

Sa'ad's (1967) circumscription of section *Acanthocladi* united all of the spined species of *Convolvulus* in a single group for the first time. The main diversity of this group is found in Western Asia and the eastern Mediterranean although the distribution is disjunct and *C. trabutianus* Schweinf. & Muschler is endemic to Morocco and Algeria and *C. caput-medusae* Lowe, endemic to Fuerteventura and Gran Canaria.

In her revision, Sa'ad (1967) considered *C. caput-medusae* and *C. trabutianus* to be conspecific. However, this proposal was vigorously challenged in a discussion on the origins of endemic Macaronesian *Convolvulus* by Mendozer-Heuer (1983) who argued that the similarity between *C. trabutianus* and *C. caput-medusae* was due to convergence and that floral characteristics clearly demonstrated the close evolutionary relationship between *C. caput-medusae* and other frutescent endemic Canarian species.

The evolution of spines in *Convolvulus* and the monophyly of section *Acanthocladi* remain to be rigorously examined within an explicit phylogenetic context. As part of a study that aims to address these issues, we present a preliminary investigation utilising data from the Internal Transcribed Spacer 2 Region (ITS 2) of nuclear ribosomal DNA. This marker has been widely used to investigate lower-level phylogenetic relationships in plants (e.g. Baldwin 1992; Gielly & al. 1996; Alice & Campbell 1999) and we use it to address four specific questions in *Convolvulus*:

1. Are *C. caput-medusae* and *C. trabutianus* closely related and possibly conspecific as proposed by Sa'ad (1967) or is *C. caput-medusae* more closely related to other Canarian endemic species as proposed by Mendozer-Heuer (1983)?
2. How many times have spines evolved in *Convolvulus*?
3. How are the spined species of *Convolvulus* related to other species in the genus?
4. What are the implications of the data for the recognition of section *Acanthocladi* and the other sections recognised by Sa'ad (1967) [i.e. *Inermes* and *Convolvulus*]?

Materials and methods

Forty species of *Convolvulus* were included in the study comprising a morphologically and geographically diverse sample from the Western Asian, Mediterranean and Macaronesian region. Of these, eight species belong to section *Acanthocladi*. *Calystegia sepium* (L.) R. Br. was selected as a placeholder for *Calystegia* which is thought to be closely related and possibly nested within *Convolvulus*. A further eight species, representing related genera of *Convolvulaceae* were included as outgroups. Table 1 lists all 49 species included in the analysis together with the source of sequence data for each.

Total genomic DNA was extracted from ~ 0,1g of dried leaf material (silica gel-dried or herbarium specimens) using a modified CTAB mini-prep extraction protocol. The extracted DNA was purified, without ethanol precipitation, using QIAquick columns (Quiagen Ltd.) and its quality and quantity was assessed by ethidium bromide stained agarose gel electrophoresis. Standard polymerase chain reaction (PCR) procedures were applied to

Table 1. Species included in the analysis and the source of sequence data (specimen details or GenBank accession number where applicable).

Taxon	Source
<i>Convolvulus acanthocladus</i> Boiss.	Mandaville 7139, Oman [BM]
<i>Convolvulus althaeoides</i> L.	Jury 17068, Morocco [RNG]
<i>Convolvulus arvensis</i> L.	Carine 302; UK [BM]
<i>Convolvulus austroaegyptiacus</i> Abdh et Sa'ad	Mandaville 1030, Saudi Arabia [BM]
<i>Convolvulus betonicifolius</i> Mill.	Davis 19318; Turkey [BM]
<i>Convolvulus boissierii</i> Steud.	Goyder 862, Spain [BM]
<i>Convolvulus buschiricus</i> Bornm.	Mandaville 4057, Saudi Arabia [BM]
<i>Convolvulus canariensis</i> L.	Stearn 1169; Canary Is.; [BM]
<i>Convolvulus cantabricus</i> L.	Fergusson et al. 6440/95, Morocco [RNG]
<i>Convolvulus caput-medusae</i> Lowe	Humphries 3095, Canary Is. [BM]
<i>Convolvulus cephalopodus</i> Boiss.	Mandaville 3717, Oman [BM]
<i>Convolvulus cneorum</i> L.	Carine 306, UK (cult) [BM]
<i>Convolvulus compactus</i> Boiss.	Davis & Dodds 18650, Turkey [BM]
<i>Convolvulus deserti</i> Hochst.	FitzGerald 3, U.A.E. [BM]
<i>Convolvulus dorycnium</i> L.	Guichard KG/Lib/422, Libya [BM]
<i>Convolvulus dryandum</i> Maire	Jury 11467, Morocco [RNG]
<i>Convolvulus farinosus</i> L.	Wood 1256, Yemen [BM]
<i>Convolvulus floridus</i> L.	Uotila & Lindberg 37098, Tenerife [BM]
<i>Convolvulus gharbensis</i> Batt. et Pit.	Jury 13738; Morocco [RNG]
<i>Convolvulus glandulosus</i> (Webb.) Sa'ad	Humphries 3047; Canary Is. [BM]
<i>Convolvulus glauorum</i> Br.-Bl. et Maire	Jury 16800, Morocco [RNG]
<i>Convolvulus glomeratus</i> Choisy	Chaudhary 3801, Saudi Arabia [BM]
<i>Convolvulus holosericeus</i> Bieb.	Davis & Hedge B29166, Turkey [BM]
<i>Convolvulus humilis</i> Jacq.	Mateos, Ramos & Villarreal 6005/95; Morocco [BM]
<i>Convolvulus hystrix</i> Vahl	Hemming 2442, Saudi Arabia [BM]
<i>Convolvulus leiocalycirus</i> Boiss.	Godman & Godman 35, Iran [BM]
<i>Convolvulus lineatus</i> L.	Jury 17613, Morocco [RNG]
<i>Convolvulus mazicum</i> Emberger et Maire	Jury 17622, Morocco [RNG]
<i>Convolvulus oleifolius</i> Desr.	Evrard 12.200, Cyprus [BM]
<i>Convolvulus oppositifolius</i> Alfarhan	Popov 68/46, Oman [BM]
<i>Convolvulus oxyphyllus</i> Boiss	Mandaville 199, Saudi Arabia [BM]
<i>Convolvulus prostratus</i> Forsk.	Wood 3427, Yemen [BM]
<i>Convolvulus pseudocantabricus</i> Schrenk	Aitchinson s.n.; Afghanistan [BM]
<i>Convolvulus sabaticus</i> Viv	Carine 301; UK (cult) [BM]
<i>Convolvulus sagittatus</i> Dulac.	Wood 2165, Yemen [BM]
<i>Convolvulus scoparius</i> L.	Kunkel 12696, Canary Is. [BM]
<i>Convolvulus siculus</i> L.	Wood A/177, Yemen [BM]
<i>Convolvulus spinosus</i> Burm.	Omer & Ghafoor 1828, Pakistan [BM]
<i>Convolvulus thymoides</i> Schwartz	Wood 3192, Yemen [BM]
<i>Convolvulus trabutianus</i> Schweinf. et Muschler	Jury 14457, Morocco [RNG]
<i>Convolvulus tricolor</i> L.	Carine 304; UK (cult) [BM]
<i>Convolvulus ulicimus</i> Boiss.	Maconochie 3396, Oman [BM]
<i>Convolvulus valentinus</i> Cav.	Else, s.n.; Morocco [BM]
<i>Convolvulus virgatus</i> Boiss.	Mandaville 7127, Oman [BM]
<i>Calystegia sepium</i> R.Br.	Carine 303; UK [BM]
<i>Argyreia nervosa</i> Boj.	GenBank (accession no AF309153)
<i>Ipomoea alba</i> Garcke	GenBank (accession no. AF256617)
<i>Ipomoea aquatica</i> Forsk.	GenBank (accession no AF110919)

<i>Ipomoea tricolor</i> Cav.	Carine 305; UK (cult) [BM]
<i>Lepistemon owariensis</i> H. Hallier ex De Wild.	GenBank, (accession no AF309157)
<i>Merremia tuberosa</i> Rendle	GenBank, (accession no AF110909)
<i>Rivea clarkeana</i> Craib.	GenBank, (accession no AF309148)
<i>Turbina cordata</i> Choisy	GenBank, (accession no AF309159)

amplify the ITS 2 region together with the 3' end of the 5.8s region and 5' end of the 26s region. The c.450bp fragment was amplified using the ITS3 forward primer (White & al. 1990) and either the ITS4 (White & al. 1990) or 26SE (Sun & al. 1994) reverse primers on a Techne Technegene Thermal Cycler programmed for 30 cycles of 1 min of denaturation at 94 °C, 3 min of annealing at 49 °C, 1 min of extension at 72 °C with a final extension of 8 min at 72 °C. Betaine (Sigma) was added to the reaction to prevent the formation of secondary structures and enhance the successful PCR of difficult templates (Henke & al. 1997). Amplified fragments were purified using QIAquick columns following the manufacturer's protocol. The concentration of pure PCR product for cycle sequencing was assessed by ethidium bromide stained agarose gel electrophoresis.

A dideoxy cycle sequencing reaction was performed on the clean PCR fragment (28 cycles: 30 sec of denaturation at 95 °C, 15 sec of annealing at 50 °C, 4 mins of extension at 60 °C) with big dye terminators (Perkin Elmer) in 10µl volumes using ITS3, ITS4 and 26SE primers on a Hybaid Omnigene Thermal Cycler. After cycling, excess dye-labelled nucleotides from the sequence reactions were removed by ethanol/sodium acetate precipitation. Sequence products were run on a Perkin Elmer ABI 377 DNA sequencer. For all taxa, chromatograms from each primer used were examined, edited and compiled into a final contig using DNASTar Lasergene Navigator. Forward strands were verified using complementary reverse strands.

Assembled sequences were aligned by eye using Se-Al (ver. 1.0a1; Rambaut 1996) prior to phylogenetic analysis. Sequences were readily aligned, except for two regions of ambiguous alignment (of 10 bp and 48 bp respectively) which were removed prior to analysis, together with regions at the 3' and 5' ends of the data set that contained a high proportion of missing data.

Phylogenetic analyses were performed using PAUP* Beta version 4.0b5 (Phylogenetic Analysis Using Parsimony; Swofford 2001). All parsimony analyses were simultaneous and unconstrained with character state changes unordered and weighted equally. To find the most parsimonious trees, a search comprising 5000 random stepwise addition replicates, using tree bisection and reconnection (TBR) branch swapping and saving all trees was completed. The relative support for different clades in the strict consensus tree was estimated using the bootstrap (Felsenstein 1985). Bootstrap values were determined from 1000 bootstrap replicates subject to full heuristic searches with simple taxon addition. Nodes supported by bootstrap values of greater than 85% were considered strongly supported.

The distribution of spines on most parsimonious tree topologies was investigated by tracing character state changes using MacClade version 3.0 (Maddison & Maddison 1992).

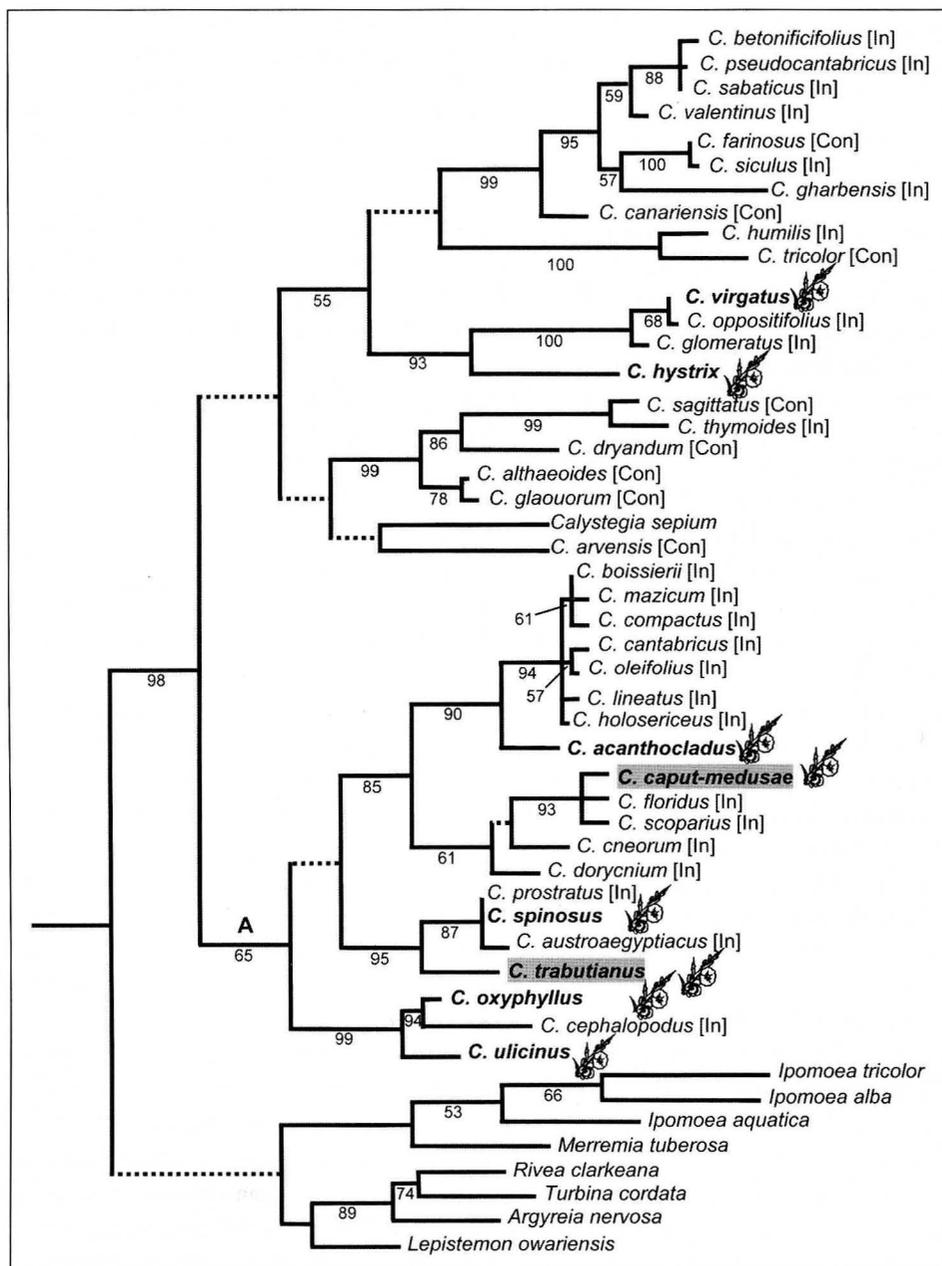


Fig 1. One of the twelve most parsimonious trees, selected at random and depicted as a phylogram. Dotted lines indicate branches which collapse in the strict consensus tree; numbers below nodes indicate bootstrap values. Species placed in section *Acanthocladi* are indicated by bold text and highlighted with the following symbol: . *Convolvulus trabutianus* and *C. caput-medusae*, which were treated as conspecific by Sa'ad (1967) are highlighted in grey. *Convolvulus* species placed in section *Inermes* and section *Convolvulus* are indicated by [In] and [Con] respectively. The clade marked 'A' is discussed in the text.

Results

The final matrix (available from the authors on request) comprised 313 characters of which 146 were variable and 110 potentially informative for parsimony analysis. Parsimony analysis of these data resulted in 12 most parsimonious trees of length 407 steps (CI [excluding uninformative characters] = 0,523; RI = 0,779).

Figure 1 shows one most parsimonious tree from the analysis on which branches collapsing in the strict consensus tree are indicated and bootstrap values given. The topology is rooted to give a monophyletic ingroup and outgroup. The analysis provides strong support for the ingroup comprising *Convolvulus* + *Calystegia sepium* (98% bootstrap). Within this group, 18 strongly supported clades are recovered although basal nodes remain poorly resolved and/or poorly supported.

The results of the analysis show that *C. caput-medusae* forms a strongly supported monophyletic group with two other Canarian endemic species of *Convolvulus*, namely *C. floridus* and *C. scoparius* (bootstrap value = 93%). *C. trabutianus* is placed in a basal position within a strongly supported clade comprising *C. spinosus*, *C. prostratus* and *C. austrae-gyptiacus* (bootstrap value = 95%). Optimising spines on the tree presented in Fig. 1 requires eight steps (with polytomies resolved to minimise the total number of steps required), demonstrating the highly homoplastic nature of this character and the polyphyly of section *Acanthocladi*. The analysis also shows sections *Inermes* and *Convolvulus* to be polyphyletic.

Discussion

The preliminary data presented in this paper provide sufficient variation (110 potentially informative sites) and contain sufficiently strong signal (with 18 ingroup nodes strongly supported) to investigate the evolution of spines in *Convolvulus* and address the taxonomic status of section *Acanthocladi*.

The results demonstrate unequivocally that the Canarian endemic *C. caput-medusae* is more closely related to other Canarian endemics (*C. floridus* and *C. scoparius*) than it is to *C. trabutianus*. Whilst *C. caput-medusae* and *C. trabutianus* are in close geographical proximity and were considered conspecific by Sa'ad (1967), the similarity between the two is a convergence in growth form in response to the similar environmental conditions in which they both occur. The results provide strong support for the hypothesis proposed by Mendoza-Heuer (1983), that the frutescent Macaronesian endemics, although morphologically very distinct, have resulted from a single Macaronesian colonisation event.

Optimising spines on to the tree presented in Figure 1 requires eight steps, clearly demonstrating the highly iterative evolution of spines in the group and the polyphyletic status of Sa'ad's section *Acanthocladi*. The independent evolution of spines from unarmed ancestors in *C. virgatus* and *C. hystrix* is strongly supported. Within clade A (Fig. 1), the optimisation of spines is equivocal as both multiple origins of spines from an unarmed ancestor and the multiple loss of spines from a spined ancestor with a secondary gain of spines in *C. caput-medusae* are equally parsimonious. Further sampling of both taxa and characters to provide a more inclusive and better supported topology will be necessary to discriminate between these two competing hypotheses to explain the evolution of spines in this group.

The polyphyly of all three sections of *Convolvulus* recognised by Sa'ad (1967) is evident from the results presented in this paper. Within *Convolvulus* there has clearly been repeated convergence upon similar growth forms from distantly related species. In light of these results, further work is now needed to re-examine the homology of those characters previously used to circumscribe groups. Detailed morphological and anatomical studies are necessary to critically evaluate the homology of spines and determine whether spines are homoplastic – i.e. the same topographically and developmentally correspondent structure evolving repeatedly within the group - or the result of convergent evolution.

The analysis should also be expanded to include not only more taxa but also other potentially informative characters. Rigorously established primary homology hypotheses for morphological and anatomical characters from a wide range of structures should be utilised together with additional molecular data to further investigate the relationships within *Convolvulus* and identify and diagnose monophyletic groups.

The preliminary results presented in this paper have demonstrated limitations of earlier investigations of the group and have highlighted a number of important areas for further investigation. An integrated molecular and morphological approach to address these issues is certain to lead to new and important insights into the evolution and classification of this widespread, species-rich and diverse group.

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