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Phylogeny of Valerianaceae based on *matK* and ITS markers, with reference to *matK* individual polymorphism

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• *Background and Aims* The monophyly of Valerianaceae and the precise delimitation of the family are not totally resolved. Our knowledge on the phylogeny of the group is only partial: on a morphological basis, some contradicting taxonomic proposals have been published, which demonstrates the difficulties in establishing a natural classification of the family and especially in proposing a relevant treatment of the large genus *Valeriana*. The aims of this study are to contribute to the phylogeny and generic delineation of the Valerianaceae on the basis of molecular data.
• *Methods* A cladistic analysis of the sequences of one plastid (*matK*) and one nuclear (ITS) molecular marker was carried out, both individually and in combination.
• *Key Results* The results of the analyses of both regions confirm that the family is monophyletic, with the exclusion of *Triplostegia*. The tribe Patrinieae is monophyletic, and the tribe Valerianeae is also a natural group. Two of the subtribes of Valerianeae, Fediinae and Centranthinae, are also monophyletic, with the exclusion of the genus *Plectritis* from Fediinae. The subtribe Valerianinae, on the other hand, is paraphyletic.
• *Conclusions* Our results confirm, for the first time on a molecular basis, the suggested paraphyly of *Valeriana* in its present circumscription, with profound nomenclatural and taxonomic implications. The correlation between molecular phylogeny and biogeography is close. In the course of the plastid DNA sequencing, a polymorphism concerning the *matK* gene was found, a fact that should be carefully evaluated in phylogenetic analyses.

Key words: Valerianaceae, Valerianeae, systematics, phylogeny, *matK*, ITS, pseudogenes.

INTRODUCTION

Valerianaceae (Dipsacales) is made up of some 400 species of almost cosmopolitan distribution, the only exceptions being Australia and the Pacific islands. Approximately 40 genera have been described. The suggested number of genera varies from one (Linné, 1753) to 16 (Graebner, 1906). Recent revisions accept 13 (Weberling, 1970) or eight genera (Eriksen, 1989).

The first genus described in the family was *Valeriana* L. (1753). As is often the case with Linnean genera, the concept of *Valeriana* was wide and included all 16 species of Valerianaceae known at that time. Miller (1754) segregated the species lacking a pappus into a new genus, *Valerianella*. Later on, three new genera were split from *Valerianella*: *Fedia* Gaertn. emend. Moench, nom. cons., *Patrinia* Juss. and *Plectritis* (Lindl.) DC. De Candolle (1815) separated the species with only one stamen from *Valeriana*, thus creating the genus *Centranthus*. Then, De Candolle (1830) segregated the species with a five-lobed accrescent calyx from *Patrinia*, and formed the new genus *Nardostachys*.

Despite the amendments by Miller (1754) and De Candolle (1815), *Valeriana* remains heterogeneous, especially because of the great diversity in the South and Central American species. Some of them lack a pappus, which could indicate that they should be placed in *Valerianella*, although none of them has ever been placed there. They have been placed in *Valeriana* or in other solely South American genera on the basis of their life history (perennials rather than annuals), ovary anatomy and inflorescence type. This heterogeneity among the American species has led to the description of many taxa, sometimes included in *Valeriana*, sometimes placed in other genera. The frequent reclassification of the species in this group indicates the unsuitability of morphology alone for clarifying systematic assignments.

Most modern authors (Graebner, 1906; Weberling, 1970) agree on the classification of Valerianaceae into three tribes, as shown in Table 1 and listed as follows.

Tribe Triplostegieae Höck, with a single genus from Asia: *Triplostegia* Wall., a genus which De Candolle (1830) placed in Valerianaceae, a position accepted by Höck (1902), Graebner (1906), Weberling (1970), Backlund and Bremer (1997) and Backlund and Nilsson (1997). However, recent molecular analyses placed *Triplostegia* as sister to the Dipsacaceae (Bell *et al*., 2001; Wen-Heng *et al*., 2003).

Tribe Patrinieae Juss., formed by two Asian genera, *Patrinia* and *Nardostachys*. Most authors agree in assigning a basal position in the family to both genera (e.g. Weberling, 1975). Molecular analyses indicate the paraphyletic nature of the tribe (Bell *et al*., 2001; Pyck *et al*., 2002).

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TABLE 1. *Classification of the Valerianaceae according to Weberling (1970)*

Tribe Patrinieae Höck
Patrinia Juss.
Nardostachys DC.
Tribe Triplostegiae Höck
Triplostegia Wall. ex DC.
Tribe Valerianeae Höck
Subtribe Fediinae Graebn. (emend. Weberling)
Plectritis (Lindl.) DC.
Valerianella Mill.
Fedia Gaertn.
Subtribe Valerianinae Graebn. (emend. Weberling)
Valeriana L. (incl. *Phuodendron* Graebn.)
Astrephia Dufr.*
Stangea Graebn.*
Aretiastrum (DC.) Spach*
Phyllactis Pers.*
Belonanthus Graebn.*
Subtribe Centranthinae Graebn.
Centranthus DC.

* Eriksen (1989) considers these exclusively South American genera as sections of *Valeriana*.

Tribe Valerianeae Höck, formed by the genera *Valeriana*, *Centranthus*, *Fedia*, *Plectritis* and *Valerianella*, with the addition of some small American genera segregated by some authors. *Valeriana s.l.* and *Valerianella*, with 200 and 50 species, respectively (Judd *et al*., 1999), are the largest genera in the family.

The tribe Valerianeae encompasses most of the diversity of the family, on morphological, geographical and numeric grounds. It is also the tribe with the most interesting and vexing taxonomic problems. They are reviewed briefly here.

The association of Fedia *and* Valerianella*: a case of incongruence between molecular data*

Fedia and *Valerianella* were grouped in Fediinae Graebn. by Weberling (1970) on the basis of some significant similarities later confirmed by several authors, namely annual habit, inflorescence type (Weberling, 1961; Ernet, 1978; Hidalgo, 1999) and genetic polymorphism of the fruits (Xena de Enrech, 1987; Xena de Enrech and Mathez, 1998; Martin and Mathez, 1991). A molecular analysis by Raymúndez *et al*. (2002) on the basis of the plastid region *atpB-rbcL* confirmed that Fediinae are a natural group: *Fedia* and *Valerianella* formed a clade at the base of Valerianeae. However, Bell *et al*. (2001) arrived at some contradictory results: their sequence analysis of the *rbcL* region also supported this association, but the analysis of the *ndhF* plastid gene sequences suggested that *Fedia* and *Valerianella* do not form a natural group. Both results were well supported, and this appears to be a clear case of molecular incongruence.

The systematic position of the genus Centranthus

Coode (1967) and Richardson (1975) pointed out a relationship between *Centranthus* and *Valeriana* because both genera share a plumose pappus. Viviani (1830) even rejected *Centranthus* as distinct from *Valeriana* because some individuals of *Centranthus* sometimes possess three stamens. Molecular analysis by Raymúndez *et al*. (2002) also indicated a close relationship between *Centranthus* and *Valeriana* but they remained uncertain as to whether they were sister groups or they should be merged. On the other hand, pollen data (Clarke, 1978) suggest a connection between Fediinae and Centranthinae Graebn. as all the genera of both subtribes show a polar thickening of the exine.

The systematic position of the genus Plectritis

This small genus from North America was first described as a section of *Valerianella*. It was later treated as a distinct genus (De Candolle, 1830) and placed near *Valerianella* in the tribe Plectritidinae Graebn. or near *Valerianella* and *Fedia* in Fediinae by Weberling (1970). On the basis of molecular data, Bell *et al*. (2001) suggested a connection between *Plectritis* and the Mediterranean genus *Centranthus*. Even though this clade is weakly supported, it is worth noting that these genera are the only ones in the family with nectar-bearing spurs.

The subgeneric classification of the Eurasian species of Valeriana*: an example of lack of morphological characters*

At the subgeneric level, the classification of *Valeriana* in subgenera and sections is difficult. Most authors agree in lumping all the Eurasian species of *Valeriana* either in one section *Euvaleriana* (Höck, 1882) or in subgenus *Valeriana* (Eriksen, 1989). The problem is that among the many morphological traits used for the species delimitation in Eurasia only a few are correlated to a degree that indicates a sectional classification. Höck (1882) was alone in suggesting a classification of all the species of *Valeriana* described in his time. However, the result of the molecular analysis by Raymúndez *et al*. (2002) was not compatible with this classification, although this analysis included only four European species.

The American species of Valeriana

The extreme diversity of the American group of species of *Valeriana* has blurred the classifications based on morphological characters. In North America, Meyer (1951) pointed out that there are two different 'provinces'. In the northern province, there are species that are also present in Eurasia (*V. dioica* L., *V. officinalis* L., etc.), or that show strong affinities to Eurasian species (*V. capitata* Pall. ex Link, *V. montana* L.). In the southern province (Mexico, Central America and the Caribbean), there are species whose affinities lie within the South American group (*V. scandens* L. and *V. clematitis* Kunth). According to pollen records (Van der Hammen, 1974, 1989; Van der Hammen and Cleef, 1983), the genus *Valeriana* colonized South America 3·5 million years ago and was among the first genera to cross the isthmus of Panama after its formation (Xena de Enrech, 1993). The subsequent explosive radiation has made impossible all attempts to establish a classification of the group on morphological grounds. A series of taxa are predominantly

South American: *Aretiastrum* DC., *Astrephia* Dufr., *Belonanthus* Graebn., *Phuodendron* Graebn., *Phyllactis* Pers. and *Stangea* Graebn. Graebner (1906) considered all to be independent genera related to *Valeriana*. In contrast, Borsini (1966) and Eriksen (1989) treated them as sections of *Valeriana*, which they divided into two subgenera: *Valeriana*, sub-cosmopolitan (Eurasia, America, Africa) and *Phyllactis*, exclusively American. However, this split is highly problematic because the only two characters used, presence versus absence of pappus (Borsini, 1966) and tetrasporangiate versus bisporangiate anthers (Eriksen, 1989), do not indicate the same groups. Raymúndez *et al*. (2002) sequenced eight species from Venezuela and Mexico, representing both subgenera of *Valeriana*, in the sense of Borsini (1966) and of Eriksen (1989). In this analysis, the subgenus *Valeriana*, as delimited by both authors, could not be considered a natural group.

This present study is part of a long survey of Valerianaceae (Xena de Enrech and Mathez, 1990; Xena de Enrech, 1992, 1993), with the recent addition of two attempts at establishing a molecular phylogeny of the family (Xena de Enrech *et al*., 2001; Raymúndez *et al*., 2002). In addition to these molecular analyses, the analysis of the *matK* and ITS regions has proved to be an efficient tool for elucidating problems related to intergeneric and interspecific relationships (Garcia-Jacas *et al*., 2001, 2002).

The goals of this study were: (*a*) to explore the correlation of the phylogeny suggested by two different genomes and the different proposals of phylogenetic history in Valerianaceae; (*b*) to discover or to verify the affinities and relationships between the genera of Valerianaceae and within the group of *Valeriana* species; and (*c*) to explore the correspondence between morphological characters commonly used in the classification of the group and the new molecular evidence.

MATERIALS AND METHODS

Plant material

The generic classification of Weberling (1970) as modified by Eriksen (1989) was followed, and representatives of all the genera of the family were included together with the two subgenera described in *Valeriana*. Outgroups were chosen among the Dipsacales (Adoxaceae, Caprifoliaceae, Dipsacaceae and Morinaceae). Both previously published and new sequences were used in the analysis. The origin of the samples and GenBank sequences accession numbers are given in Table 2. Thirty-six new sequences of *matK* and 14 new sequences of ITS were examined. For technical reasons, it was not possible to obtain more sequences of the ITS region (see Results). However, the information provided by these ITS sequences justifies their inclusion in this article (see Discussion). Other sequences were obtained from GenBank (Table 2).

DNA extraction, amplification and sequencing

Total genomic DNA was extracted following the miniprep procedure of Doyle and Doyle (1987) as modified by Soltis et al. (1992) and Cullings (1992), from silica-gel-dried leaves collected in the field, from plants cultivated in the Botanical Garden of Montpellier, or from fresh leaves of plants cultivated in the Botanic Institute of Barcelona.

cpDNA matK *gene strategies.* The first 960 base pairs (bp) at the 5′ end were sequenced because this region includes most of the variability in *matK* (Khidir and Hongping, 1997). Partial *matK* was amplified by PCR with the primers *trn*K-710F and *matK*-1848R (Johnson and Soltis, 1995).

PCR products were cleaned with a QIAquick PCR Purification Kit (Qiagen Inc., Valencia, CA) and sequenced with *trn*K-710F, *matK*-1168R, *matK*-1412R, *matK*-1470R and *matK*-1848R (Johnson and Soltis, 1995) as sequencing primers. Direct sequencing of the amplified DNA segments was performed using the BigDye Terminator Cycle Sequencing v2·0 (PE Biosystems, Foster City, CA). Nucleotide sequencing was carried out at the Serveis Científico-Tècnics of the University of Barcelona on an ABI PRISM 3700 DNA analyser (PE Biosystems).

nrDNA ITS region strategies. The ITS1, ITS2 and 5·8S gene (the ITS region) were amplified and sequenced together. The ITS region was amplified by PCR with 1406F (Nickrent *et al*., 1994) and ITS1 (White *et al*., 1990) as forward primers, and ITS4 (White *et al*., 1990) as the reverse primer. PCR products were purified using the QIAquick PCR Purification Kit (Qiagen Inc.). Both strands were sequenced with 1406F or ITS1 as forward sequencing primers and ITS4 as reverse primer. Direct sequencing of the amplified DNA segments was performed as for the *matK* region.

Phylogenetic analysis

Nucleotide sequences were edited with Chromas 1·56 (Technelysium Pty, Tewantin, Australia). The *matK* DNA sequences were aligned visually by sequential pairwise comparison (Swofford and Olsen, 1990) and were translated into proteins with GeneJockey (Biosoft, Cambridge, UK) to verify the absence of internal stop codons. Due to the high level of variability of the ITS sequences, the alignment was checked with ClustalX (Thompson *et al*., 1997) and adjusted manually. Data matrices are available on request from the corresponding author. Parsimony analysis involved heuristic searches conducted with PAUP version 4·0b4a (Swofford, 1999) using Tree Bisection Reconnection (TBR) branch swapping with character states specified as unordered and unweighted. All most-parsimonious trees (MPT) were saved. To locate islands of most-parsimonious trees (Maddison, 1991), we performed 100 replicates with random taxon addition, also with TBR branch swapping. Trees lengths, consistency index (CI) and retention index (RI) are always given excluding uninformative characters. To verify the length of the branches, a neighbour-joining analysis of the *matK* sequences was also conducted with PAUP version 4·0b4a (Swofford, 1999).

TABLE 2. *Origin of the material, herbaria where the vouchers are deposited and GenBank accession numbers (PS: pseudogene sequences accession number)*

Species	Voucher	ITS accession	*matK* accession
Abelia chinensis R. Br.	Montpellier Botanical Garden, France (MPU)		AY310461
Centranthus angustifolius (Mill.) DC.	France: Hautes-Alpes, *Hidalgo 428 (*MPU)	AY310446	AY310484
Centranthus calcitrapae (L.) Dufr.	France: Hérault, La Gardiole, *Mathez 1032 (*MPU)		AY310483
Centranthus lecoqii Jord.	France: Hérault, près Saint-Guilhem le désert, *Mathez 1076 (*MPU)	AY310447	AY310485
Centranthus ruber DC.	France: Gard, Vauvert, *Hidalgo 503* (MPU)	AY310448	AY310487
Centranthus trinervis (Viv.) Bég.	France: Corse du Sud, Bonifacio, Chévreloup Botanical Garden, *Fridlender s. n.* – 1997 (MPU)	AY310495	AY310488
Dipsacus mitis D. Don	Bell *et al.* (2001)		AF446917
Fedia cornucopiae (L.) Gaertn.	Bell *et al.* (2001)		AF446923
Fedia graciliflora Fisch. & C. A. Meyer	Montpellier Botanical Garden, France (MPU)	AY310449	AY310489 PS
Fedia pallescens (Maire) Mathez	Morocco: Mehdyia, *El-Oualidi s. n.* – 1998 (MPU)	AY310450	
Linnaea borealis L.	Bell *et al.* (2001)		AF449611
Lonicera etrusca Santi	France: Hérault, environs de Saint Martin de Londres, *Mathez s. n.* (MPU)		AY310460
Morina longifolia Wall.	Bell *et al.* (2001)		AF446915
Nardostachys jatamansi (D. Don) DC.	Bell *et al.* (2001)		AF446920
Patrinia intermedia (Hornem.) Roem. & Schult.	Caputo P, Cozzolino S, De Castro O, Moretti A. (unpublished)	AJ426557 AJ426558	
Patrinia saniculaefolia Hemsl.	North Korea: Mt Sorak, Pyunggang Botanical Garden, 25–2000 (MPU)		AY310462
Patrinia triloba Miq.	Bell *et al.* (2001)		AF446921
Patrinia villosa (Thunb.) Juss.	Japan: Sanbe-san, *Hiroshi s. n.* (MPU)	AY310493	AY310463
Plectritis congesta (Lindl.) DC.	USA: Oregon, *C. Roché s. n.* – 2001 (MPU)		AY310486
Sambucus nigra L.	Montpellier Botanical Garden, France (MPU)		AY310458
Scabiosa africana L.	Caputo P, Cozzolino S, De Castro O, Moretti A. (unpublished)	AJ426543 AJ426544	
Scabiosa columbaria L.	Bell *et al.* (2001)		AF446918
Scabiosa uniseta Savi	Caputo P, Cozzolino S, De Castro O, Moretti A. (unpublished)	AJ426547 AJ426548	
Symphoricarpos albus (L.) S. F. Blake	Montpellier Botanical Garden, France (MPU)		AY310459
Symphoricarpos orbiculatus Moench	Bell *et al.* (2001)		AF446904
Triplostegia glandulifera Wall. ex DC.	Bell *et al.* (2001)		AF446919
Valeriana albonervata Robinson ex Seaton	Mexico: Tamaulipas, Missouri Botanical Garden, *Barrie & Cowan 1400* (MEXU, MO, TEX)		AY310474
Valeriana apula Pourr.	France: Pyrénées Orientales, Nohèdes, *Hidalgo 494* (MPU)		AY310472
Valeriana bractescens (Hook.) Höck	Venezuela: Mérida, Mucubají, *Mathez & Xena de Enrech s. n.* (VEN)	AY310451	AY310479
Valeriana celtica L.	Italy: Aosta, Cogne, *Hidalgo 503* (MPU)	AY310494	
Valeriana dioica L.	France: Gard, vallon du Bonheur, *Mathez & Raymúndez s. n.* – 1998 (MPU)		AY310468
Valeriana hardwickii Wall.	Nepal: Dolpa, *Thomas Yat 4* (MPU)		AY310464
Valeriana jatamansi Jones	Pakistan: Hazara, *Thomas Yat 3* (MPU)		AY310469 AY310490 PS
Valeriana longiflora Willk.	Spain: Aragón, Ibars de Noguera, *Garnatje*, *Hidalgo 500 & Luque* (MPU)		AY310482
Valeriana montana L.	France: Savoie, col du Galibier, *Hidalgo 447* (MPU)		AY310471 AY310492 PS
Valeriana officinalis L. subsp. *tenuifolia* Schübler & Martens	France: Gard, Massif de l'Aigoual, *Mathez 1046* (MPU)		AY310467
Valeriana officinalis L.	Caputo P, Cozzolino S, De Castro O, Moretti A. (unpublished)	AJ426559 AJ426560	
Valeriana parviflora (Trevir.) Höck	Venezuela: Mérida, páramo Mucuchies, *Xena de Enrech 1359* (VEN)	AY310452	AY310480
Valeriana phylicoides (Turcz.) Briq.	Venezuela: Mérida, *Mathez & Xena de Enrech s. n.* (VEN)		AY310478
Valeriana pilosa Ruiz & Pav.	Venezuela: Táchira, páramo Batallón, *Xena de Enrech 1346* (VEN)		AY310481
Valeriana pyrenaica L.	France: Ariège, *Hidalgo 479* (MPU)	AY310453	AY310470 AY310491 PS
Valeriana rosaliana C. A. Meyer	Venezuela: Táchira, páramo El Rosal, *Xena de Enrech 1356* (VEN)		AY310477
Valeriana saliunca All.	France: Savoie, col du Galibier, *Hidalgo 464 (*MPU)		AY310473
Valeriana scandens L.	Venezuela: Mérida, *Xena de Enrech s. n.* (VEN)	AY310454	AY310475
Valeriana tachirensis Xena	Venezuela: Táchira, *Xena de Enrech s. n.* (VEN)	AY310455	AY310476
Valerianella eriocarpa Desv.	Italy: Palermo,Monte Gallo, Palermo Botanical Garden 766–1999 (MPU)		AY310466
Valerianella locusta L.	France: Hérault, Montpellier, *Mathez 1035* (MPU)	AY310456	AY310465
Viburnum tinus L.	Spain: Barcelona, Montjuïc, *Hidalgo 497* (MPU)		AY310457

Bootstrap (BS) and Bremer support (Bremer, 1994) or 'decay index' (DI) were carried out to obtain support estimates of the nodes of the trees selected. Bootstrap analysis was performed (Felsenstein, 1985) using 1000 replicates of a heuristic search with the default options. The Bremer support of each node was conducted by successive analysis using the clade constraint approach as discussed in Morgan (1997) with 100 replicates. ACCTRAN (accelerated transformation) character-state optimization was used for all illustrated trees. Three

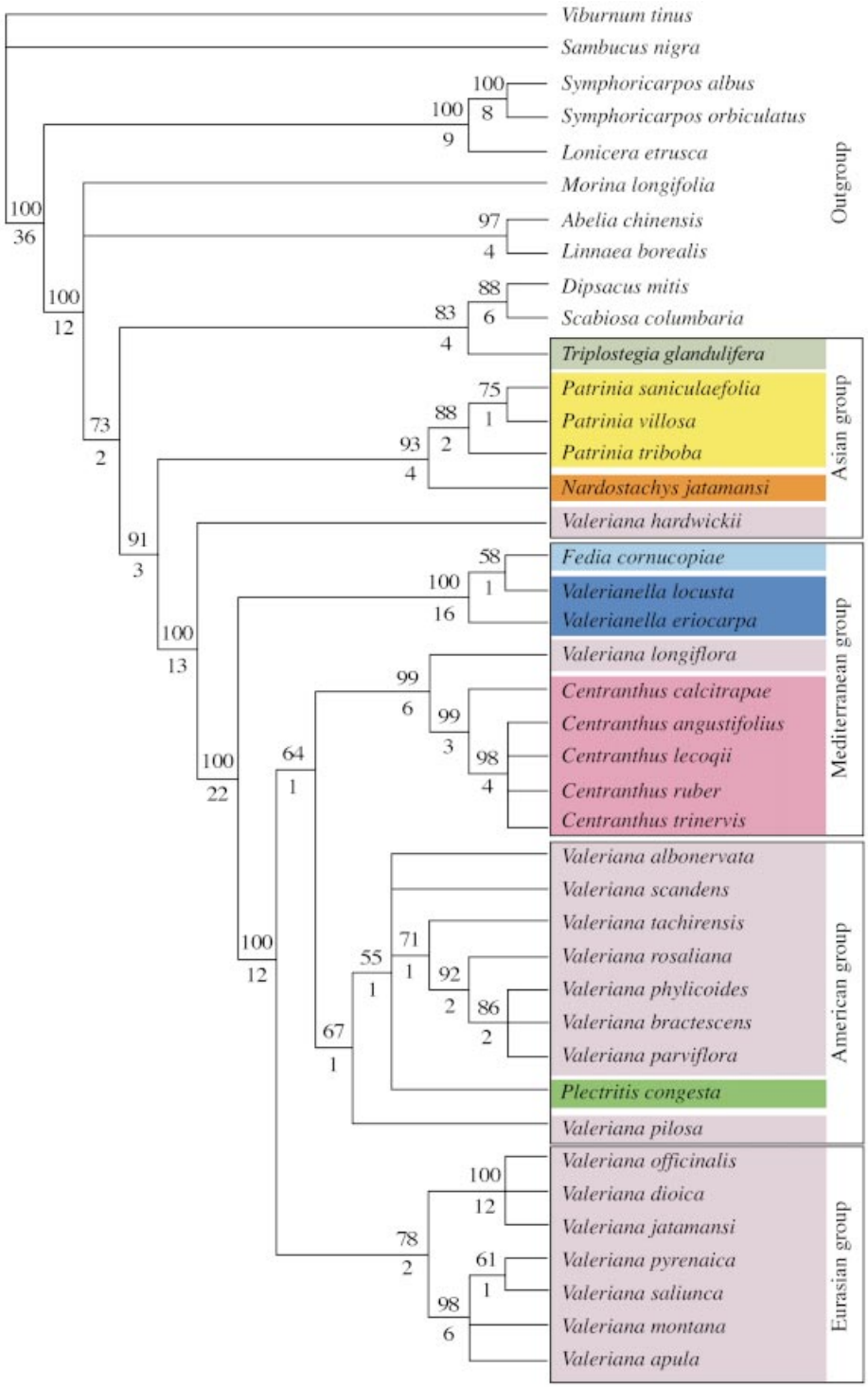

FIG. 1. Strict consensus tree of the eight most parsimonious trees generated by the *matK* matrix. Numbers above branches represent bootstrap values and numbers below indicate Bremer supports.

TABLE 3. *Comparison of results from the ITS and* matK

Data set	*matK*	ITS
Number of taxa	41	18
Total characters	960	522
Informative substitutions	228	187
Number of MPTs	8	6
Number of steps	556	357
Consistency index (CI)	0·7115	0·7437
Retention index (RI)	0·9294	0·8321
Mean pairwise distances, ingroup (%)	11·6	25·2

The consistency and homoplasy indexes are calculated excluding uninformative characters.

different codings of indels were tried: missing data, total omission and fifth base.

RESULTS

matK *analysis*

The *matK* alignment for 41 taxa consisted of 960 positions and contained 228 phylogenetically informative substitutions and 45 phylogenetically informative gapped positions. The major insertion/deletion event (indel) appears as a deletion of 39 bp (position 197–235) in all the sequences of *Valeriana* (apart from *V. hardwickii* Wall.), *Centranthus* and *Plectritis*. Mean pairwise distance (as calculated by PAUP) within the ingroup varied from 0 % [between the Andean species *Valeriana bractescens* (Hook.) Höck, *V. parviflora* (Trevir.) Höck and *V. phylicoides* (Turcz.) Briq. and between *Centranthus angustifolius* (Mill.) DC., *C. ruber* DC. and *C. trinervis* (Viv.) Bég.] to 11·6 % (between *Patrinia triloba* Miq. and *Valerianella locusta* L.). Mean pairwise distance between ingroup and outgroup varied from 2·7 % (between *Abelia chinensis* R. Br. and *Nardostachys jatamansi* (D. Don) DC.) to 17·3% (between *Sambucus nigra* L. and *Valerianella locusta*).

The only change resulting from the different treatments of indels concerned *Triplostegia*, which was placed as sister to Dipsacaceae with strong support when the indels were coded as missing data or fifth base. When the indels were coded as 'total omission' this node collapsed in the analysis. The reason for this is that the informative positions coincide with a gap in one species and are therefore eliminated by this method. Finally, it was decided to code the indels as 'missing data'.

The parsimony analysis yielded eight MPTs of 556 steps in one island. The strict consensus of all trees is shown in Fig. 1; the CI was 0·7115 and the RI was 0·9294 (Table 3).

Valerianaceae (excluding *Triplostegia*) are monophyletic (BS = 91%, DI = 3) (Fig. 1). The association of *Nardostachys* and *Patrinia* and their position as sister to the remainder of the family are strongly supported (BS = 93%, DI = 4 and BS = 100%, DI = 13, respectively). *Patrinia* is monophyletic with moderate support (BS = 88%, DI = 2). The Asian species *Valeriana hardwickii* is sister to the remainder of Valerianeae (BS = 100%, DI = 22). Within this group, *Fedia* and *Valerianella* form a strongly supported clade (BS = 100% and DI = 16) and are strongly supported as sister group to a clade consisting of *Valeriana* (excluding *V. hardwickii*), *Centranthus* and *Plectritis* (BS = 100% and DI = 12). These genera share the previously mentioned 39 bp deletion. Even though we coded indels as missing data (and thereafter lost the phylogenetic information conveyed by this large indel), support for this branch is extremely high. This clade is divided into two groups. The first group is a moderately supported clade formed only by species of *Valeriana* (BS = 78%, DI = 2), divided into two robust clades, one with *Valeriana officinalis* L. subsp. *tenuifolia* Schüber & Martens, *V. dioica* and *V. jatamansi* Jones (BS = 100%, DI = 12), and the other with *V. pyrenaica* L., *V. saliunca* All., *V. montana* and *V. apula* Pourr. (BS = 98%, DI = 6). The second group is a weakly supported (BS = 64%, DI = 1) clade formed by the genera *Centranthus* and *Plectritis* and some *Valeriana* spp. This heterogeneous clade is divided into two branches: a well-supported clade (BS = 99%, DI = 6) with *Valeriana longiflora* Willk. and the genus *Centranthus*, which appears to be monophyletic (BS = 96%, DI = 3) with *C. calcitrapae* (L.) Dufr. sister to the rest of the species (BS = 98%, DI = 4). The second clade is weakly supported (BS = 67%, DI = 1), and encompasses the American species and the genus *Plectritis*. Finally, the American group is poorly resolved, the only exception being the clade formed by *Valeriana parviflora*, *V. bractescens*, *V. phylicoides* and *V. rosaliana* C. A. Meyer with moderate support (BS = 92%, DI = 2), with *V. rosaliana* as sister to the three other species (BS = 86%, DI = 2).

For *Valeriana montana*, *V. pyrenaica* and *V. jatamansi*, two different *matK* sequences were determined, starting with the same amplification product obtained always from a single individual. The two *matK* sequences were separated by using different sequencing primers. The translation of these sequences into proteins revealed the existence of many stop codons in one of the sequences for each one of the three species. A phylogenetic analysis including both types of sequences shows all the sequences with stop codons grouped together within Valerianaceae. The possibility of long-branch attraction was excluded by carrying out a neighbour-joining analysis (Fig. 2). Our conclusion is that the sequences with stop codons correspond to non-functional genes or pseudogenes. Regarding *Fedia graciliflora* Fisch. & C. A. Meyer, only a single *matK* sequence was obtained which groups together with the *Valeriana* pseudogenes; this sequence also contains many stop codons and thus corresponds to a pseudogene. Our hypothesis is confirmed by the results of Bell *et al*. (2001), who sequenced the *matK* region of *Fedia cornucopiae* (L.) Gaertn., and this sequence is placed in a conventional position in our analysis.

Wendel and Doyle (1998) explained the presence of pseudogenes by means of an ancestral polymorphism that was retained throughout speciation processes. As pseudogenes are not homologous to normal genes, we have removed them from the final analyses involving the *matK* gene.

ITS analysis

Our DNA extractions were often contaminated with alien DNA that, at least in one case (*Valeriana tripteris* L.), was

identified as the endophytic fungus *Colletotrichum destructivum* O'Gara. The idenfication was carried out by comparison with the sequences from GenBank (ITS sequences for approx. 15 species of fungi were obtained). This contamination was so extensive that it precluded further sequencing of the ITS region without resorting to cloning.

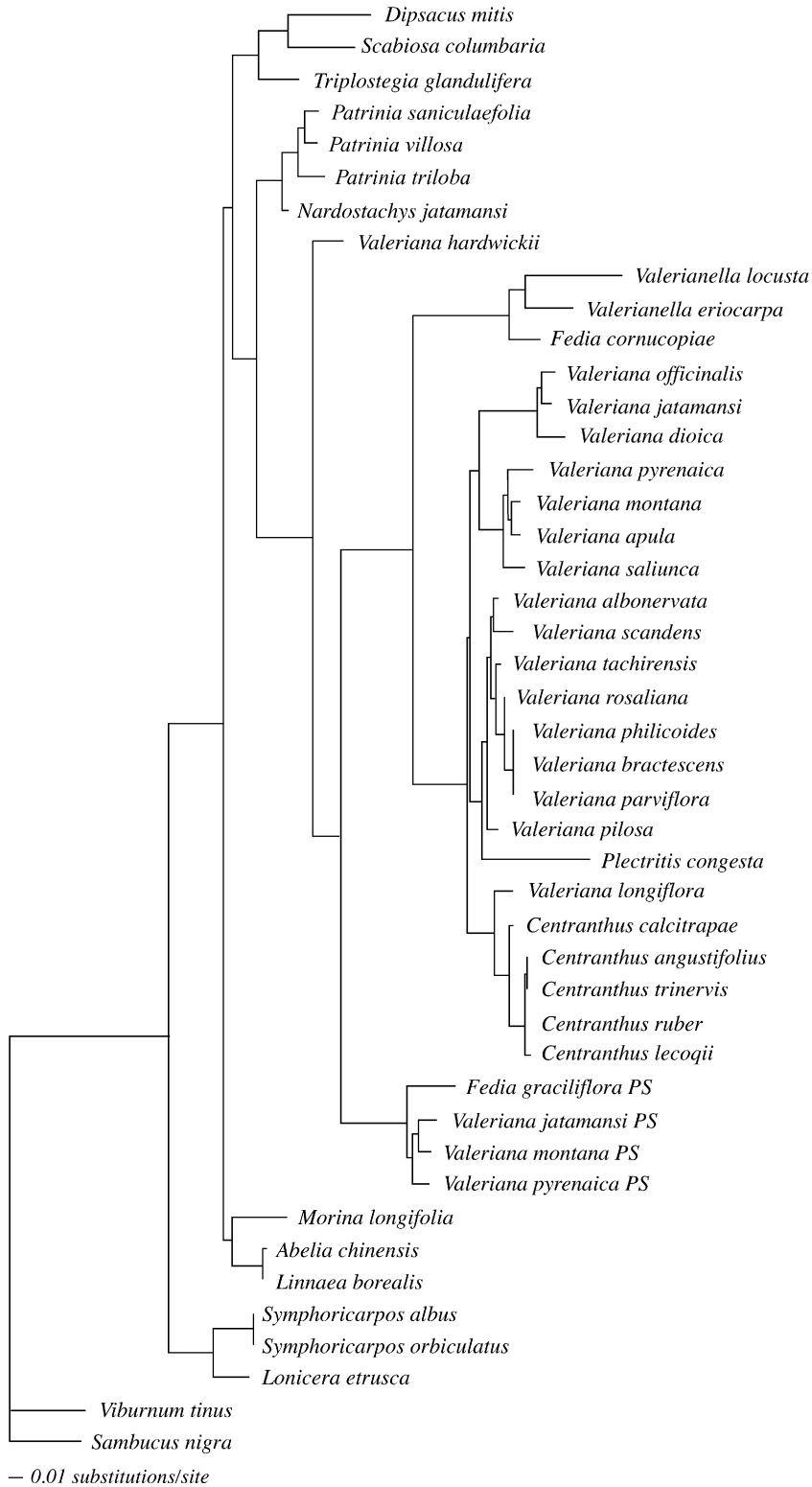

FIG. 2. Neighbour-joining phylogram generated by the *matK* matrix including the pseudogene (PS) sequences.

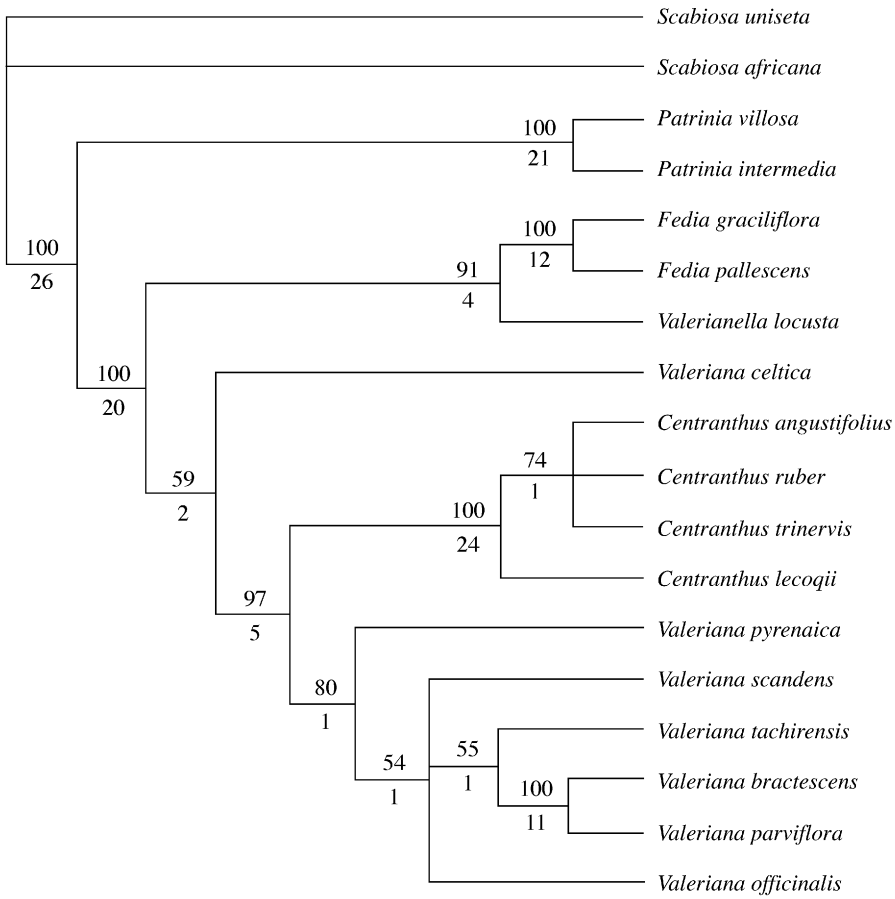

FIG. 3. Strict consensus tree of the six most parsimonious trees generated by the ITS matrix. Numbers above branches represent bootstrap percentages and numbers below indicate Bremer supports.

The ITS alignment for 18 taxa consisted of 522 positions and contained 187 phylogenetically informative substitutions. The mean pairwise distance (as calculated by PAUP) within the ingroup varied from 0 % (between *Valeriana bractescens* and *V. parviflora*, and between *Centranthus trinervis* and *C. angustifolius*) to 25·2 % [between *Centranthus angustifolius* and *Patrinia intermedia* (Hornem.) Roem. & Schult.]. Mean pairwise distance between the ingroup and outgroup varied from 16·9 % [between *Scabiosa africana* L. and *Patrinia villosa* (Thunb.) Juss.] to 25·5 % (between *Scabiosa uniseta* Savi and *Centranthus angustifolius*).

The ITS alignment shows numerous indels, often combined with substitutions of nucleotides among the studied species. We coded these indels as missing data because this seems to be the more adequate approach where the sequences contain important insertion–deletion zones (Raymúndez *et al*., 2002).

The parsimony analysis yielded six MPT of 357 steps. The tree obtained is shown in Fig. 3; the CI was 0·7437 and the RI was 0·8321 (Table 3).

Patrinia is monophyletic (BS = 100%, DI = 21) and is strongly supported as sister to *Valerianeae* (BS = 100%, DI = 20). *Fedia* and *Valerianella* form a robust clade (BS = 91%, DI = 4), sister to an unsupported group formed by *Valeriana* and *Centranthus* (BS = 59%, DI = 2). *Valeriana celtica* L. is sister to the remainder of *Valeriana* and *Centranthus* (BS = 97%, DI = 5). *Centranthus* is monophyletic (BS = 100, DI = 24), and sister to a weakly supported group formed by *Valeriana* (excluding *V. celtica*). In this group, only the clade formed by *V. parviflora* and *V. bractescens* is strongly supported (BS = 100, DI = 11).

DISCUSSION

Our results are basically coincident with the biogeography of Valerianaceae, with four groups being identified: Asian, Mediterranean, Eurasian and American.

The Asian group is formed by the genera *Patrinia* and *Nardostachys*, with the addition of *Valeriana hardwickii*. As was the case in the molecular analysis by Bell *et al*. (2001), *Triplostegia glandulifera* Wall. ex DC. falls outside Valerianaceae with the species of Dipsacales used as the outgroup. This position contradicts not only morphological evidence, but also chemical data: *Triplostegia* contains valepotriates, a family of chemical compounds only known

from Valerianaceae (Backlund and Moritz, 1998). Further studies are needed to clarify this contradiction.

Patrinia and *Nardostachys*, with strong morphological affinities, are sister to the remainder of the family in a well-supported clade (tribe Patrinieae) in the *matK* analysis (Fig. 1). Bell *et al*. (2001) and Pyck *et al*. (2002), on the other hand, placed *Nardostachys* as derived in relation to *Patrinia*.

The unexpected position of *Valeriana hardwickii*, as sister to the remainder of Valerianeae, makes the genus *Valeriana* paraphyletic. However, *V. hardwickii* is an Asian species, and Valerianaceae are generally considered to have an Asian origin (Höck, 1902; Eriksen, 1989). Our results also suggest an Asian origin for the Valerianeae. This hypothesis needs confirmation, especially because of the profound taxonomic and nomenclatural implications of the paraphyly of the genus *Valeriana*.

The Mediterranean group is made up of two different clades. The first group is formed by the genera *Fedia* and *Valerianella* and conforms to the subtribe Fediinae *sensu* Weberling (1970), with the exclusion of *Plectritis*. Both ITS (Fig. 3) and *matK* (Fig. 1) analyses strongly support this association, as did those of *rbcL* (Bell *et al*., 2001) and *atpB-rbcL* (Raymúndez *et al*., 2002). The ITS analysis supports the monophyly of *Fedia* as for *atpB-rbcL* (Raymúndez *et al*., 2002). The subtribe Fediinae is sister to the rest of the tribe Valerianeae (excluding *V. hardwickii*).

A second clade is formed by a monophyletic *Centranthus* and *Valeriana longiflora*. This unexpected association opens up an avenue for elucidating the origin of *Centranthus*, which could be merged into *Valeriana*. The most important morphological trait shared by these taxa is the long corolla tube. Relationships of this group and the Eurasian and American ones are obscure in the analyses.

The Eurasian group is formed by two robust clades in *matK* analyses. One includes *Valeriana officinalis* subsp. *tenuifolia*, *V. dioica* and *V. jatamansi*, and the other one *V. montana*, *V. pyrenaica*, *V. apula* and *V. saliunca*. Neither of these groups coincides with the classification suggested by Höck (1882). We find here once again the lack of useful morphological traits among the Eurasian species of *Valeriana*. To date, we have been unable to detect any character for defining either group on morphological grounds. The ITS analysis places *V. celtica* as sister to Valerianinae, which makes *Valeriana* paraphyletic.

The American group is made up of species of *Valeriana* from both American subcontinents with the addition of a North American species of *Plectritis*. This group of species is not supported, or only weakly supported, in both the *matK* (Fig. 1) and ITS (Fig. 3) analyses. The position of *Plectritis* within the American clade has never been suggested before. The low resolution of this group in the *matK* tree does not allow any inference on its precise position, but the exclusion of *Plectritis* from the subtribe Fediinae seems to be confirmed. Its origin should be sought within *Valeriana*.

Our results confirm the paraphyly of *Valeriana* in its present delimitation, for four reasons. Firstly, *V. hardwickii* is placed at the base of the tribe Valerianeae. Secondly, *V. longiflora* is sister to *Centranthus*. Thirdly, *Plectritis* is firmly nested in the clade of American *Valeriana* spp. Fourthly, *V. celtica* is sister to the subtribe Valerianinae. Certainly, a redefinition of the subtribal and generic boundaries and some nomenclatural changes will be necessary to reconcile the classification of the family with this new evidence. However, our sampling of species and sections of *Valeriana* is not complete: American species are not well represented, which makes our results somewhat provisional. Prior to any changes, more comprehensive representation of the genus is necessary.

The *matK* region has proved to be an excellent tool: the *matK* tree is finely resolved and the nodes are well supported, both in deep and terminal branches. The exceptions (polytomies and weakly supported branches in the American and Eurasian clades) must correspond to incomplete sampling of these groups, which it is intended to correct in further studies, or insufficient variation in *matK* especially in the cases of rapid radiation and recent speciation.

Regarding the ITS results, for the first time a nuclear region has been used for phylogenetic analysis of Valerianaceae, whilst five different plastid ones have already been used. The ITS results are concordant with the *matK* phylogeny, and confirm the monophyly of the subtribe Fediinae which was unclear in the *ndhF* analysis (Bell *et al*., 2001).

Discovery of pseudogenes in this group brings new and interesting perspectives. The literature includes only a few examples of plastid DNA polymorphisms crossing species boundaries (Wendel and Doyle, 1998). According to GenBank data, *matK* pseudogenes are reported here for the first time within Dicotyledones. Moreover, species possessing these pseudogenes in our analysis form a monophyletic group within Valerianaceae. This implies that species with pseudogenes share a common polymorphic ancestor. Why was this pseudogene only found in a few descendants of this taxon? Two hypotheses could be put forward. It could be the result of incomplete lineage sorting (by which the polymorphism of the common ancestor was lost in most species). Alternatively, all descendants of the polymorphic ancestor could be polymorphic, but we have sequenced only one type of *matK* sequence for each species (the efficient one for most of them).

In view of the excellent results obtained with the *matK* region, a new analysis involving this gene should be considered for the purposes of resolving some problems revealed by our research. Perhaps the most interesting questions that remain open are the origin of *Centranthus*, the position of *Plectritis* within *Valeriana*, and the reasons for the unexpected placement of *V. hardwicki* and *V. celtica*.

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