

Molecular phylogeny of the genera *Digitalis* L. and *Isoplexis* (Lindley) Loudon (Veronicaceae) based on ITS- and *trnL-F* sequences

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Abstract. To investigate phylogenetic and biogeographic relationships all species of *Digitalis* and *Isoplexis* and one species of the outgroup genera *Antirrhinum* and *Globularia* each were analyzed using nuclear ITS and plastid *trnL-F* sequences. Phylogenetic trees resulting from separate analyses were highly congruent. Combined analysis revealed two major lineages, which mark an early split in the genus *Digitalis*. While sections *Digitalis*, *Frutescences* and *Globiflorae* appear monophyletic, sect. *Tubiflorae* is polyphyletic and sect. *Macranthae* should be expanded due to paraphyly. Our results provide evidence that all species of the genus *Isoplexis* have a common origin and are embedded in *Digitalis*. *Isoplexis* therefore should be reduced to sectional rank. The phylogenetic placement combined with ecomorphological characters indicates that *Isoplexis* may be a bird-pollinated Tertiary relict. Results are discussed in the context of biogeography, chemotaxonomy and morphology.

Key words: *Digitalis*, *Isoplexis*, molecular phylogeny, ITS, *trnL-F*, biogeography, ornithophily.

Introduction

The genus *Digitalis* L., commonly known as the “foxglove” is member of the Veronicaceae (sensu Olmstead et al. 2001). All *Digitalis*

species, are biennial or perennial herbs, rarely small shrubs with simple, alternate leaves, which are often crowded in basal rosettes. Flowers are zygomorphic and arranged in terminal, bracteate racemes. The calyx is equally five-lobed and shorter than the corolla tube. The corolla, with a cylindrical-tubular to globose tube, is often constricted at the base and the limb is more or less two-lipped. The upper lip is usually shorter than the lower, which is spotted or veined inside. The species are native throughout northern Africa, Europe and parts of Asia (Werner 1965) with main centers of diversity in the western and eastern Mediterranean. Several *Digitalis* species (e.g. *D. purpurea*, *D. lanata*) are potent sources of cardiac glycosides and since the discovery of the beneficial effects by Withering (1785) they have been used therapeutically for the treatment of cardiac insufficiency.

The genus *Digitalis* was first described by Linné (1753) who recognized five species. These included *Digitalis canariensis*, which is currently assigned to the Macaronesian genus *Isoplexis* and according to Werner (in Luckner and Wichtl 2000) was first raised to generic rank by Loudon (1829) prior to Bentham’s

publication in 1835. It comprises the four species *Isoplexis sceptrum* (L. f.) Loudon endemic to Madeira, *I. canariensis* (L.) Loudon restricted to Tenerife and La Palma, *I. isabelliana* (L.) Loudon which is confined to Gran Canaria and *I. chalcantha* Svent. & O'Shann., a taxon described from Gran Canaria in 1968.

According to Werner (1965) the four species of *Isoplexis* are united by several synapomorphies such as growth form (candelabra shrub), corolla colour (orange-yellow), shape of corolla tube (ventral side concave), ratio of upper lip to corolla tube (1:1 or 1:2), ratio middle limb of lower lip to upper lip (< 1) and position of inflorescences (clearly separated from the vegetative parts by long internodia). Additionally all taxa show a presence of specific cardenolids and polysaccharides such as D-Canarose and Canarobiose (Luckner and Wichtl 2000). Based on the morphological characters combined with a restricted distribution to some of the Macaronesian Islands, *Isoplexis* is considered to be a relict of an ancient Tertiary flora (Sunding 1979). In view of the high chromosome number ($2n = 56; 8x$) Bramwell (1972) regarded *Isoplexis* as a typical palaeoendemic element. However, contrary to other genera (e.g. *Aeonium*, *Echium*, *Argyranthemum*, *Tolpis*, *Sonchus*) which have undergone a remarkable adaptive radiation on the Macaronesian Islands, *Isoplexis* is only split in four species.

The two most recent and complete taxonomic treatments of *Digitalis* (Ivaniva 1955 and Werner 1960, 1961, 1964, 1965, 1966) are quite contradictory. Ivaniva's (1955) conclusions to accept 36 species found no major acceptance as her definition and circumscription of species lay within very narrow bounds. Alternatively, the most widely accepted treatment (e.g. Heywood 1972a, Meusel et al. 1978) is that of Werner (1960, 1961, 1964, 1965, 1966), who recognized only 19 species. Based on phytogeographical and morphological features Werner (1965) proposed five more or less natural groups of species. A short characterization of these sections is given in Table 1. According to Heywood (1972b) sect. *Grandi-*

florae is a nomen illegitimum and consequently sect. *Macranthae* is the new valid name for this section, a nomenclatural change also accepted by Werner (pers. comm.). Additionally a couple of biosystematic studies dealt with the taxa of sect. *Digitalis* (Hinz et al. 1986; Hinz 1987a,b; 1989a,b; 1990a,b) placing them within a *Digitalis purpurea* aggregate.

Within *Digitalis* there is a remarkable variation of flower features, especially concerning size, shape and colour of the corolla. The latter ranges from white, yellow and cream to pink and rose. In addition maculations often occur over parts of the corolla and could be interpreted as adaptations to various insect pollinators. As hybridization has been documented for some species there is speculation that this process has played an important role in the evolution of *Digitalis* (e.g. Werner 1965). The hypotheses on the origin and phylogenetic relationships of *Digitalis* and *Isoplexis* have been subject to an ongoing controversy. Werner (1965) postulated a common ancestor which he supposed to be an element of the Tethyan flora. Based exclusively on morphological and biogeographical data Humphries (1979) suggested a monophyletic origin of the two genera from a common ancestor from the Canary Islands. Perez de Paz and Roca (1982) considered that *Digitalis* had probably evolved from *Isoplexis*. They suggested that the Ibero-Moroccan *D. obscura* subsp. *laciniata* and the Canarian *Isoplexis chalcantha* could be transitional taxa between the two genera.

Werner (1965) suggested that the diversification of *Digitalis* started in the early Tertiary (ca. 50 mya) from "ancient *Frutescentes*", which were widespread in the Mediterranean region and have retained a set of plesiomorphic characters shared with the ancestors of *Isoplexis*. According to Werner (1965) these are: candelabrous shrubby habit, flowers with a campanulate corolla tube, almost equal corolla limbs and a weak reduction of the upper lip.

Recently the phylogenetic relationships of the tribe Digitaleae, along with 24 tribes of the Scrophulariaceae s.l. and 15 other families of

Table 1. Short characterization of the sections of *Digitalis* accepted by Werner (1965)

Sect. <i>Frutescentes</i> Benth.	Small shrubs; all parts glabrous (except corolla); leaves coriaceous and shiny; pedicels long (> 5 mm); flowers in short, almost one-sided racemes; corolla orange-yellow or rusty-brown with tubular-campanulate tube.
Sect. <i>Digitalis</i> L.	Perennial or biennial herbs; often densely pubescent; leaves more or less rugose; pedicels very long (> 8 mm); flowers in one-sided racemes; corolla tube campanulate, purple, pale pink or white, usually spotted or pointed inside.
Sect. <i>Grandiflorae</i> Benth. em. Werner (syn. <i>Macranthae</i> Heywood)	Perennial or biennial herbs; sparsely pubescent; leaves more or less smooth; pedicels short (< 5 mm); flowers in one-sided racemes; corolla tube campanulate-ventricose, ochre-yellow, dark veined beneath.
Sect. <i>Tubiflorae</i> Benth.	Perennial herbs; glabrous or pubescent; pedicels short (< 5 mm); racemes with flowers pointing to one side or in all directions; corolla tube tubular to slightly ventricose.
Sect. <i>Globiflorae</i> Benth.	Predominantly perennial herbs; leaves coriaceous, smooth, entire; pedicels short (< 5 mm); racemes with flowers pointing in all directions; corolla tube inflated-globose.

the Lamiales were explored using the plastid genes *rbcL*, *ndhF* and *rps2* by Olmstead et al. (2001). This molecular study indicated that the Scrophulariaceae as traditionally defined are not monophyletic. One of the strongly supported major clades includes all representatives of Bentham's (1846) tribe Digitaleae (*Digitalis*, *Hemiphragma* and *Veronica*) along with parts of other tribes of Scrophulariaceae and the conventional families Callitrichaceae, Globulariaceae, Hippuridaceae and Plantaginaceae. According to the tree topology and the taxa associated with this large clade the oldest valid family name Veronicaceae (Wettstein 1891) was resurrected.

A first provisional attempt in using sequence data as a tool to implement studies of molecular systematics in *Digitalis* was made by Carvalho and Culham (1997, 1998) based on a low sampling of taxa. An approach to elucidate relationships within the genus *Digitalis*, based on PCR-generated RAPD markers was undertaken by Nebauer et al. (2000), who studied inter-specific variation among six taxa of *Digitalis*. The species relationships revealed by this study were consistent with those obtained using morphological affinities by Werner (1965).

In this paper a molecular phylogenetic study of the genera *Digitalis* and *Isoplexis*

based on both nuclear (ITS) and chloroplast (*trnL-F*) markers is presented. Our main aims were: (1) to test the monophyly of the genera *Digitalis* and *Isoplexis* (2) to infer the phylogenetic relationships between the genera and sections, particularly to test previous systematic treatments and the hypothesis of Werner (1965) and (3) to reexamine morphological character evolution and biogeographic patterns in the context of the molecular results.

Materials and methods

Plant material. All recognized *Digitalis* and *Isoplexis* species, as well as most subspecies of *Digitalis* (except *D. purpurea* subsp. *amandiana* (Samp.) Hinz, *D. cariensis* subsp. *carionensis* Boiss. ex Jaub. et Spach emend. Werner and *D. cariensis* subsp. *lamarckii* (Ivan.) Werner), were included in the analysis. *Antirrhinum majus* (Veronicaceae) and *Globularia trichosantha* (Veronicaceae) were selected as outgroups based on the phylogeny of Olmstead et al. (2001).

Table 2 lists all taxa used in this study and summarizes sources, voucher specimen data and GenBank accession numbers. Voucher specimens are deposited at the Botanische Staatssammlung Munich (M) or in the private collections Melzheimer (Marburg) and Bräuchler (Munich). Two samples of *D. lutea* subsp. *australis* from different sites were analyzed.

Table 2. List of taxa investigated in our analysis with voucher specimen data (locality (abr.), date, collector, coll.-nr.) and GenBank accession number. BGM = Bot. Garten München, M = Herbarium Bot. Staatssammlung München, HB = Herbarium Bräuchler, Melz = Herbarium Prof. V. Melzheimer

Taxon	Source /Voucher	Accession-nr. ITS / <i>trnL</i> -F
<i>Globularia trichosantha</i> Fisch. & C.A. Mey.	BGM, HB, 5.10.2002	AY591287/AY591321
<i>Antirrhinum majus</i> L.	BGM, HB, 5.10.2002	AY591288/AY591322
<i>Isoplexis sceptrum</i> (L.f.) Loudon	M, Madeira, 10.9.1986, Hertel 33616	AY591268/AY591302
<i>I. isabelliana</i> (L.) Loudon	HB, Gran Canaria, Tejada Visitors-Centre, 2001, Dittrich	AY591270/AY591304
<i>I. calcantha</i> Svent. & ÓShann.	HB, Gran Canaria, Bot. Garden Tafira Alta, 2001, Dittrich	AY591269/AY591303
<i>I. canariensis</i> (L.) Loudon	BGM, HB, 1.11.2000	AY591271/AY591305
<i>I. canariensis</i> f. <i>trichomana</i>	HB, Tenerife, La Lagunetas, path to Erjos, 2001, Dittrich	AY591272/AY591306
<i>Digitalis obscura</i> subsp. <i>obscura</i> L.	M, Spain, 28.5.1988, Podlech 44082	AY591273/AY591307
<i>D. obscura</i> subsp. <i>laciniata</i> (Lindl.) Maire	M, Spain, Type locality, 13.5.1979, Lopez	AY591274/AY591308
<i>D. laevigata</i> subsp. <i>laevigata</i> Waldst. et Kit.	M, 2.8. 1976, Podlech 28182	AY591281/AY591315
<i>D. laevigata</i> subsp. <i>graeca</i> (Ivan.) Werner	M, Greece, 12.07.1982, Podlech 37716	AY591280/AY591314
<i>D. ferruginea</i> subsp. <i>ferruginea</i> L.	M, Croatia (Dalmatia), 12.6.1976	AY591279/AY591313
<i>D. ferruginea</i> subsp. <i>schischkinii</i> (Ivan.) Werner	M, W Caucasian Mts., 10.7.1979, Vasak	AY591278/AY591312
<i>D. nervosa</i> Steud. et Hochst. ex Benth.	M, Iran, 15.7.1971, Rechinger 43415	AY591283/AY591317
<i>D. cariensis</i> subsp. <i>trojana</i> (Ivan.) Werner	M, Turkey, Anatolia, 2.7.1993, Nydegger	AY591282/AY591316
<i>D. lanata</i> subsp. <i>lanata</i> Ehrh.	M, Greece, 21.6.1985, Lippert 20684	AY591285/AY591319
<i>D. lanata</i> subsp. <i>leucophaea</i> (Sibth. et Sm.) Werner	M, N Greece, 6.2000, Franke et Iosifidou	AY591284/AY591318
<i>D. parviflora</i> Jacq.	M, Spain, 21.7.1990, Zubizarreta 38324	AY591286/AY591320
<i>D. subalpina</i> var. <i>subalpina</i> Br.-Bl.	Melz, Morocco, high Atlas, 25.6.1986, Lichius 86/257	AY591275/AY591309
<i>D. lutea</i> subsp. <i>australis</i> (Ten.) Arcang.	M, Italy (Tuscany), 16.7.1990, Nepi et Aldobrandi	AY591277/AY591311
<i>D. lutea</i> subsp. <i>australis</i> (Ten.) Arcang.	M, France (Corsica), 11.6.1989, Aurich	AY591265/AY591299
<i>D. subalpina</i> Br.-Bl. (syn. <i>D. lutea</i> var. <i>atlantica</i>)	M, Morocco, 26.8.1951, Rauh 500	AY591276/AY591310
<i>D. lutea</i> subsp. <i>lutea</i> L.	M, France, 26.7.1990, de Retz 89971	AY591266/AY591300
<i>D. viridiflora</i> Lindl.	M, Greece, 22.6.1985, Lippert 20751	AY591262/AY591296
<i>D. ciliata</i> Trautv.	M, E Caucasian Mts., 26.7.1979, Vasak et Esvandzia	AY591264/AY591298
<i>D. grandiflora</i> Mill.	M, Germany, 3.7.1994, Förther 8329	AY591261/AY591295

Table 2. (continued)

Taxon	Source /Voucher	Accession-nr. ITS / <i>trnL</i> -F
<i>D. davisiana</i> Heyw.	M, Turkey, 22.07.1947, Davis 13629	AY591267/AY591301
<i>D. atlantica</i> Pomel	Melz, Algeria, 23.06.1989, Melzheimer	AY591263/AY591297
<i>D. minor</i> L. (syn. <i>D. dubia</i> Rodr.)	M, Spain, Mallorca, 17.06.1974, Durigneat 74E685	AY591255/AY591289
<i>D. thapsi</i> L.	M, Spain, 23.6.1988, Amich y Sanchez	AY591256/AY591290
<i>D. mariana</i> subsp. <i>mariana</i> Boiss.	M, Spain, 4.6.1973, Podlech & Lippert 25009	AY591259/AY591293
<i>D. mariana</i> subsp. <i>heywoodii</i> (Silva et Silva) Hinz	M, Spain, 18.5.1981, Malato-Beliz et al. 16341	AY591260/AY591294
<i>D. purpurea</i> subsp. <i>purpurea</i> L.	M, Germany, 5.7.1993, Schuhwerk 93/179	AY591257/AY591291
<i>D. purpurea</i> subsp. <i>toletana</i> (Font Quer) Hinz	M, Spain, 21.05.1994, Nydegger 32962	AY591258/AY591292

DNA isolation. Total DNA was extracted from fresh material and/or herbarium specimen (approximately 0.5g leaf material per sample) using the NucleoSpin Plant-Kit (Macherey-Nagel, Germany) following the manufacturer's protocol with an additional phenol/chloroform extraction to remove disturbing secondary compounds. The DNA was dissolved in 30 µl elution buffer (10 mM Tris/HCl) and checked for quality on a 1% agarose-gel. A standard amount of 1 µl of the dissolved DNA was used for amplification.

Amplification. Two noncoding regions, ITS (ITS1, 5.8S rDNA, ITS2) from nuclear DNA and *trnL*-F from chloroplast DNA, were chosen for phylogenetic analysis. The markers were amplified from total DNA via the polymerase chain reaction (PCR) using Taq polymerase (Boehringer) and the primer pairs aITS1 (5'-AGAAGTCCACTGAA CCTTATC-3') and aITS4 (5'- CGCTTCTCCAG-ACTACAATTC-3') (designed by Meimberg). For amplification of the *trnL*-F region primers according to Taberlet et al. (1991) were used. All PCR amplifications were carried out in a MWG thermocycler (Primus). For ITS the following program was chosen: (1) 94 °C for 2 min 30 sec, (2) 40 cycles at 94 °C for 30 sec, 54 °C for 30 sec, 72 °C for 1 min 15 sec and (3) a terminal extension phase at 72 °C for 10 min. For *trnL*-F each cycle consisted of 94 °C for 30 sec, 55 °C for 30 sec, 72 °C for 1 min 30 sec, the other steps remained unchanged. The PCR pro-

ducts were purified with QuantumPrep Gel Slice Kit (Bio-Rad, USA) for sequencing on GATC 1500 direct blotting system and with MinElute Gel Extraction Kit (Qiagen, Germany) for sequencing on an ABI 377 automated sequencer.

Sequencing. For most ITS sequences Cycle Sequencing was performed on a GATC 1500 direct blotting system as described elsewhere (Meimberg et al. 2001). The DNA sequences were read and corrected several times. All *trnL*-F and some ITS sequences were gained from sequencing on an ABI 377 automated sequencer following manufacturer's protocol. Both markers were sequenced bidirectionally using the same primer pairs as for amplification. To close gaps in the ITS sequence of *D. parviflora* the additional internal primers aITS3 (5'-CATCGATGAAGAACGTAG-3' designed by Meimberg) and ITS2 (White et al. 1990) were used.

Phylogenetic analysis. The sequences were aligned and edited base by base using the multi sequence alignment editor GENEDOC (Nicholas and Nicholas 1997) with gaps coded as additional characters. The resulting data matrix was subsequently analyzed using PAUP* version 4.0b10 (Swofford 2002). All heuristic searches were carried out with the following settings: SIMPLE addition, TBR branch-swapping, MULPARS, COLLAPSE=min for *trnL*-F and combined data set, COLLAPSE=max for ITS data set and ACC-TRAN optimization and character states specified

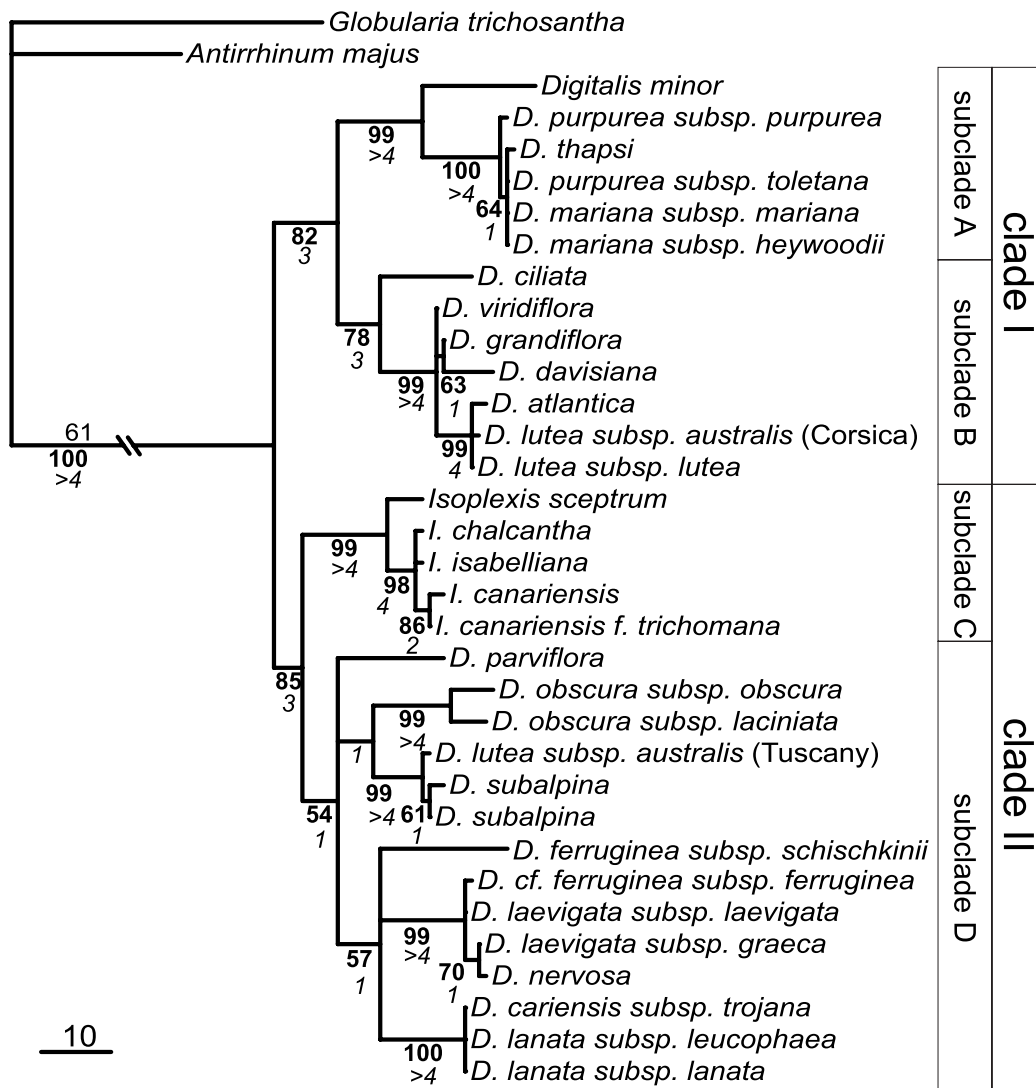


Fig. 1. Strict consensus tree of four most parsimonious trees based on ITS sequence data of *Digitalis* and *Isoplexis* shown as phylogram. Bootstrap values/decay values (italic) are indicated below each branch. Bootstrap values are expressed as percentages of 1000 bootstrap replications. Branches with support less than 50% are shown as polytomies. Clade and subclade names are indicated at the right margin

as unordered and equally weighted. Bootstrap values from 1000 replicates (Felsenstein 1981) and decay-indices were calculated. As decay-indices the additional number of steps at which a branch is collapsed is given. Strict consensus trees were constructed from all most parsimonious trees. The sequences reported in this study are available from GenBank; their accession numbers are provided in Table 2. The alignment is available from the authors upon request. To assess character congruence between the two data sets a partition homogeneity test as implemented in PAUP* 4.0b10

(Swofford 2002) was performed with the same heuristic search settings as used in the phylogenetic analysis of the ITS region. The test included 1000 replicates with a maxtrees setting of 100 per replicate.

Results

Analysis of ITS. The ITS matrix included 25 taxa of the genus *Digitalis*, two of these from two localities each, four species and one forma of *Isoplexis* and in addition *Globularia tricho-*

santha and *Antirrhinum majus* as outgroups. The length of ITS1 and ITS2 in the taxa surveyed was 219-222 bp and 201-204 bp, respectively. The 5.8S rDNA sequences each were 164 bp long with four variable sites, three of them potentially phylogenetically informative. In the multiple alignment of the entire data set part of 18S (16 bp) and 26S rDNA (113 bp) were included. The aligned sequences (outgroup taxa excluded) had 722 positions, of which 576 (79.8%) were constant, 146 (20.2%) were variable, including 108 (15%) parsimony-informative characters. The G+C content varied from 45.7-53.2% in ITS1, 40.2-47.6% in 5.8S rDNA and 43.8-47.6% in ITS2. The alignment resulted in 11 inferred insertion/deletion events (indels) of which eight were potentially phylogenetically informative. Six of the indels occurred in ITS1 and five in ITS2. All indels were from one to two bp long.

Phylogenetic analysis (excluding uninformative characters) resulted in eight most parsimonious trees, each 360 steps long (CI=0.78; RI=0.88). The topology of the ITS strict consensus tree (L=365, CI=0.72, RI=0.87; phylogram see Fig. 1) indicates that *Digitalis* is separated into two major clades with high bootstrap support (bts=82%/85%; decay value d=3/3). Within clade II a sister relationship between all taxa of the Macaronesian genus *Isoplexis* and a lineage of *Digitalis* is apparent.

Clade I of *Digitalis* shows two monophyletic groups, the first (subclade A, bts=99%, d>4) including *Digitalis purpurea* subsp. *purpurea* and subsp. *toletana*, *D. thapsi*, *D. mariana* subsp. *mariana* and subsp. *heywoodii* and *D. minor*. Within this group *D. minor*, with maximum bootstrap support is sister to some taxa revealing almost identical ITS sequences (*D. purpurea* subsp. *toletana*, *D. mariana* subsp. *mariana* and *D. mariana* subsp. *heywoodii*).

Within subclade B (bts=78%; d=3) *D. ciliata*, is separated by a high bootstrap value (bts=99%; d>4) and is sister to the remaining taxa. Close relationships are indicated between *D. atlantica*, *D. lutea* subsp. *lutea* and *D. lutea* subsp. *australis* (Corsica) (bts=99%; d=4)

and there is weak support (bts=63%; d=1) for a branch comprising *D. grandiflora* and *D. davisiana*.

Within clade II all taxa of the genus *Isoplexis* are shown in a well defined group (subclade C, bts=99%; d>4). *I. sceptrum* is highly supported (bts=98%; d=4) sister to the remaining taxa *I. isabelliana*, *I. chalcantha* and *I. canariensis*.

Subclade C with high support (bts=85%; d=3) is sister to a weakly supported lineage of *Digitalis* (subclade D, bts=54%; d=1). The latter comprises *D. parviflora* as terminal taxon and two further distinct groups. Within one of these lineages (bts<50%, d=1) a strong linkage between *D. obscura* subsp. *obscura* and subsp. *laciniata* (bts=99%; d>4) as well as between *D. lutea* subsp. *australis* (Tuscany) and *D. subalpina* (bts=99%; d>4) is indicated. The second lineage is also weakly supported (bts=57%; d=1). Within this alliance the close relationships between *D. cariensis* subsp. *trojana*, *D. lanata* subsp. *lanata* and *D. lanata* subsp. *leucophaea* (exhibiting identical sequences) are well supported (bts=100%; d>4). A further sharply distinct subclade (bts=99%; d>4) includes *D. ferruginea* subsp. *ferruginea*, *D. laevigata* subsp. *laevigata* and subsp. *graeca* along with *D. nervosa*. The latter two monophyletic groups together with *D. ferruginea* subsp. *schischkinii* form a polytomy.

Analysis of *trnL-F*. The *trnL-F* matrix included the same set of taxa as the ITS analysis. The length of the partial *trnL*(UAA)-Intron sequences ranged from 446 to 451 bp, the 3' exon was constantly 50 bp long and the partial 3' spacer had a length of 330-399 bp. The aligned *trnL-F* region (outgroup taxa excluded) covered 909 positions, of which 877 (96.4%) were constant and 30 (3.6%) variable. 12 characters (1.3%) were parsimony informative. The G+C content varied from 35.3-36.3% in the *trnL*(UAA)-Intron, 44.0% in the 3' exon and 32.3-34.0% in the 3' spacer.

The alignment resulted in 12 indels, six of which were potentially phylogenetically informative. One of the indels (position 720-726

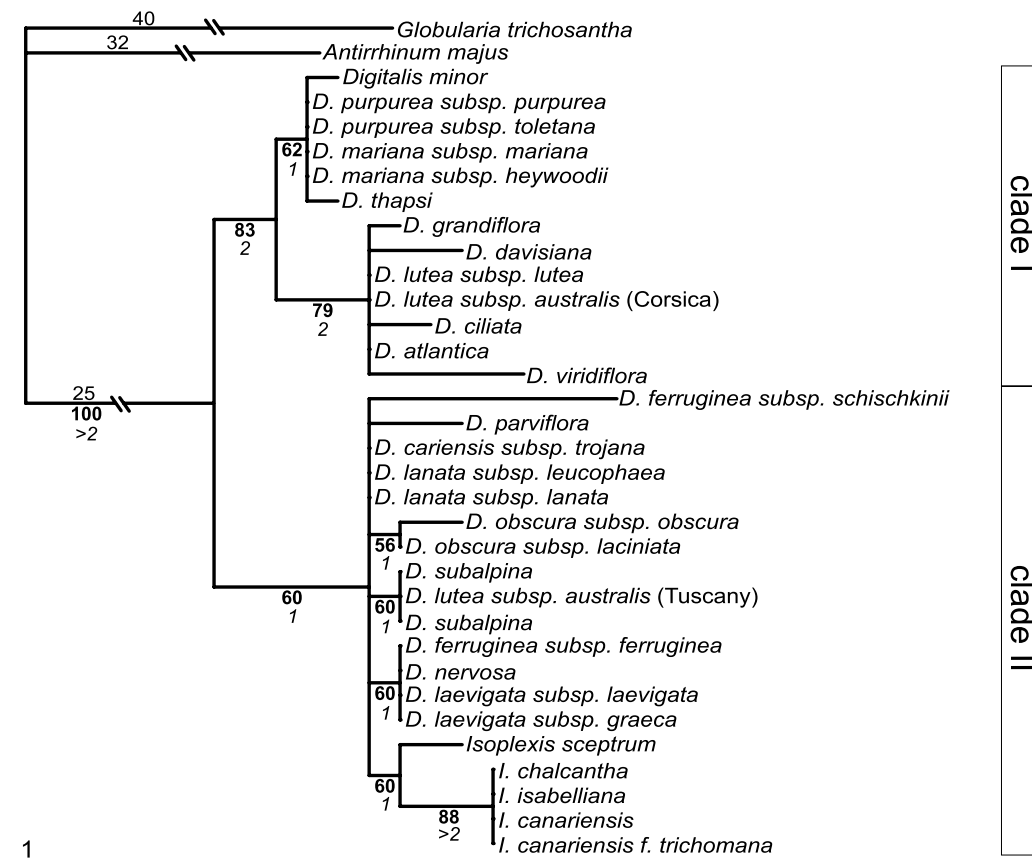


Fig. 2. Strict consensus tree of 32 most parsimonious trees revealed from maximum parsimony analysis of *trnL-F* sequences of *Digitalis* and *Isoplexis* shown as phylogram. Bootstrap values/decay values (italic) are indicated below each branch. Bootstrap values are expressed as percentages of 1000 bootstrap replications. Branches with support less than 50% are shown as polytomies. Clade names are indicated at the right margin

in the alignment) came out as a probable homoplasy shared by *Isoplexis canariensis* and *Digitalis lutea* subsp. *australis*, its encoding resulting in the collapse of a branch comprising *D. lutea* subsp. *australis* from Tuscany and *D. subalpina* var. *subalpina* (data not shown). Maximum parsimony analysis yielded 32 trees (L = 144, CI = 0.91, RI = 0.89) resulting in a strict consensus tree with L = 148, CI = 0.91 and RI = 0.89.

Concerning the subdivision of *Digitalis* into two major clades (bts = 83%/60%; d = 2/1) the *trnL-F* phylogram (Fig. 2) is in accordance with the topology obtained from analysis of the ITS matrix, though less resolved than the latter. Due to the lower amount of variable and potentially phylogenetically informative positions respectively, bootstrap and decay values for all major

clades in the *trnL-F* tree are lower. Nevertheless the taxa of *Isoplexis* are also shown as a monophyletic group (bts = 60%; d = 1). The remaining taxa of *Digitalis* clade II form an unresolved polytomy. Closer relationships are only assumed between *D. obscura* subsp. *obscura* and subsp. *laciniata*, (bts = 56%; d = 1) as well as between four taxa with identical sequences (*D. ferruginea* subsp. *ferruginea*, *D. laevigata* subsp. *graeca*, *D. laevigata* subsp. *laevigata* and *D. nervosa*) (bts = 60%; d = 1).

Clade I (bts = 83%; d = 2) again consists of two distinct subclades, representing subclade A (bts = 62%; d = 1) and subclade B (bts = 79%; d = 2) of the ITS phylogeny. Both subclades each represent an unresolved polytomy.

Combined matrix. A partition homogeneity test, used to evaluate character congruence,

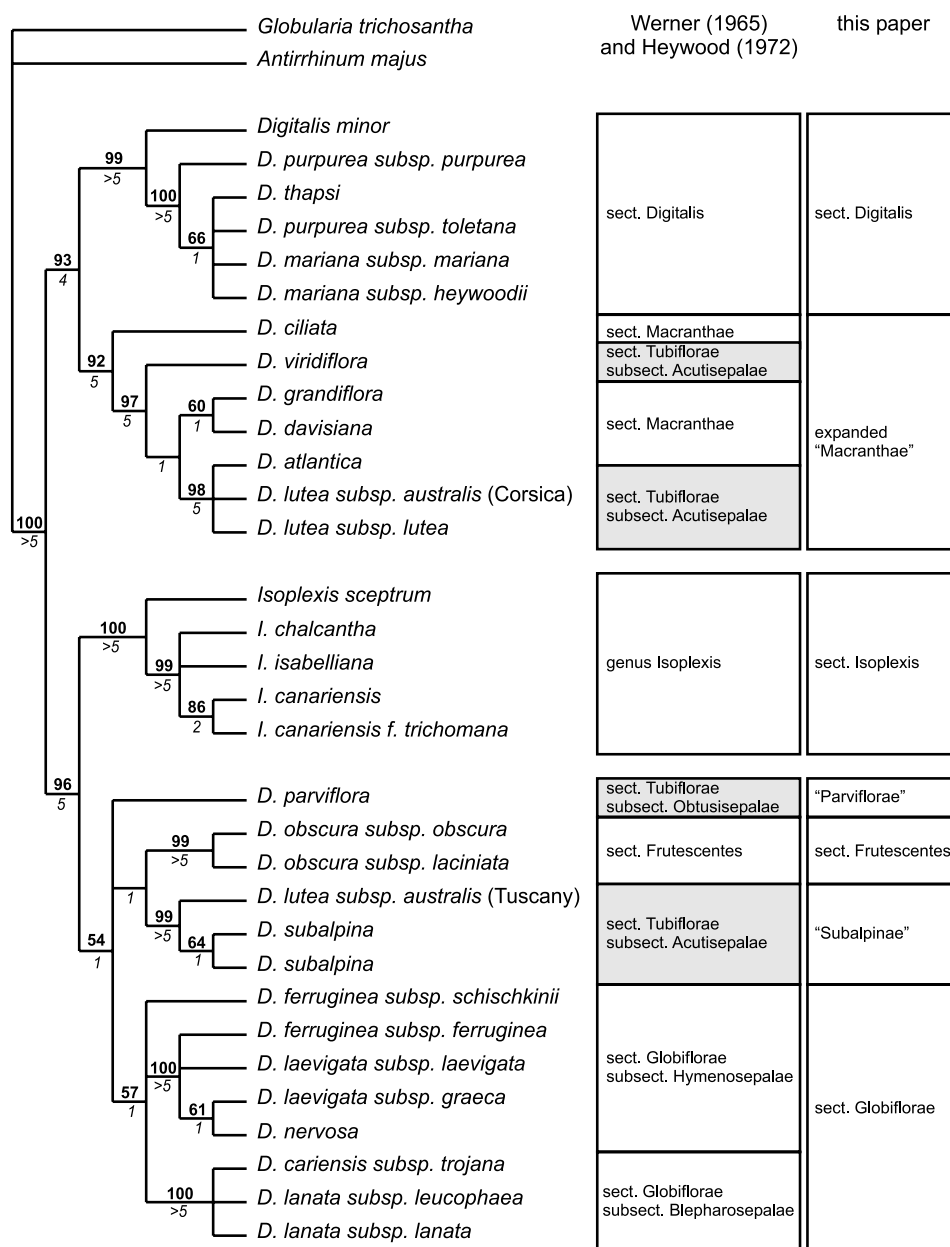


Fig. 3. Strict consensus tree of the four equally most parsimonious trees, based on the combined sequence data set of ITS and *trnL-F*, shown as cladogram. Right margin sectional classification according to Werner (1965) and Heywood (1972), farthest right subdivision according to our molecular analysis (this paper). Tree length $L = 510$, $CI = 0.82$, $RI = 0.88$. Numbers above the branches indicate bootstrap support, numbers below decay values

indicated that our data from two distinct marker regions were homogeneous ($p = 0.98$), thereby justifying the combined analysis of both loci. The combined matrix included the identical set of taxa as single marker matrices.

It consisted of 1664 positions, 176 of which (10.6%) were variable and 120 (7.2%) potentially parsimony informative. The combined analysis resulted in four most parsimonious trees compared to *trnL-F* or ITS alone (32 and

8 trees, respectively). The strict consensus tree was 510 steps long with CI=0.82 and RI=0.88 (cladogram see Fig. 3).

In general, there are no contradictions between the topology of the trees obtained from the combined analysis and those from analyses of the separate matrices (Figs. 1, 2). As a result the cladogram of the combined analysis has a topology which is almost entirely congruent with that of the ITS phylogeny. The only difference (position of *D. viridiflora*, see Figs. 1, 3) lacks proper bootstrap support. Bootstrap and decay values in most cases are higher than in the single analyses. It is of particular interest that the paraphyly of *Digitalis* is even stronger supported (bts=96%; d=5) in the combined analysis, with species of *Isoplexis* (bts=100%; d>5) embedded between the two monophyletic groups of *Digitalis* (clade I/subclade D, Fig. 3).

Discussion

Taxonomic implications and biogeography. The taxa of subclade A (Fig. 1) exclusively are members of *Digitalis* sect. *Digitalis* (Werner 1965) as shown in Fig. 3, or the *D. purpurea* aggregate sensu Hinz (1990b) respectively. As in the phylogeny presented here sect. *Digitalis* is shown as a natural monophyletic group, it probably could be maintained in the traditional circumscription (Werner 1965).

The section shows a center of diversity on the Iberian Peninsula with *D. minor*, endemic to the Balearic Islands, indicated as sister to the other species. Based on results from palaeohistory and geology of the western Mediterranean basin, Hinz (1990b) considered the origin of *D. minor* to be probably pre-Messinian (>5–6 mya).

On the contrary, *D. purpurea* subsp. *toletana*, *D. mariana* subsp. *mariana* and subsp. *heywoodii* as well as *D. thapsi*, altogether closely restricted endemics on the Iberian Peninsula, are completely identical in both ITS and *trnL-F* sequences. From these *D. purpurea* subsp. *purpurea* can only be distin-

guished by one autapomorphic indel. Consequently the Iberian taxa of the *D. purpurea* complex (excluding *D. minor* and *D. purpurea* subsp. *purpurea*) seem to have a recent origin and probably evolved via areal fragmentation, strongly influenced by glaciation during the Pleistocene. Due to the high similarity of these taxa it is favourable that *D. purpurea* subsp. *amandiana* (Samp.) Hinz and var. *nevadensis* (Kze.) Amo, both also from the Iberian Peninsula and not included in this study, would not show much differences on the molecular level. The status of the geographical more distant varieties of *D. purpurea*, var. *gyspergerae* (Rouy) Burnat ex Briq. from Sardinia and Corsica and var. *mauretanica* Humb. et Maire ex Maire et Emb. from NW Africa, are currently under investigation. Whilst the taxa of the Iberian Peninsula included here appear as local endemics with areas of hybridization (Hinz 1990a), *D. purpurea* subsp. *purpurea* has colonized large regions, primarily the atlantic western and central parts of Europe (Meusel et al. 1978). Due to human activities this taxon was probably introduced to Madeira (Perez de Paz and Roca 1982), Iceland, North and South America, New Zealand and SE Australia and frequently became naturalized. Hinz (1990b) suggested that *D. purpurea* subsp. *purpurea* occupied its extended area in Europe in the last postglacial period, thus explaining the homogeneity of this taxon outside the Iberian Peninsula. The assumption of Ivaniva (1955) that *D. purpurea* subsp. *purpurea* is a Tertiary relict is not supported by molecular data. With exception of *D. minor* the close affinities within the *D. purpurea* alliance as postulated by Werner (1965) and Hinz (1990b) were fully confirmed in the molecular phylogeny. The latter author also suggested *D. minor* to be the most isolated taxon in the group.

In a sister group relationship to sect. *Digitalis* a monophyletic group of taxa appears (subclade B, Fig. 1), which Werner (1965) assigned to sect. *Macranthae* and sect. *Tubiflorae* (Fig. 3). It is of special interest that two widespread members *D. viridiflora* and *D. lutea*

of the former sect. *Tubiflorae* subsect. *Acutisepalae* are nested within sect. *Macranthae* in the molecular phylogeny, which thus is shown as a paraphyletic group. Due to the dissociation of *D. lutea* subsp. *australis* and the positions of *D. subalpina* and *D. parviflora* in our tree topology, sect. *Tubiflorae* is indicated as polyphyletic. As this section should be completely rejected from a taxonomical point of view, sect. *Macranthae* probably should be expanded (Fig. 3) and newly circumscribed.

With exception of *D. atlantica* and *D. lutea* these taxa have a center of diversity in the eastern Mediterranean. The most basal taxa within this group are *D. ciliata*, endemic to the western Caucasus where it occurs at elevations up to 2400 m, and *D. viridiflora* which occurs from the southern part of the Balkan Peninsula to the Rhodope mountains. The most widespread taxon within this section is *D. grandiflora* extending from central to eastern Europe and southwards to the Apennine and Balkan Peninsulas. This species is closely related to *D. davisiana* an endemic taxon from southern Anatolia (Davis 1978). *D. atlantica*, an endangered taxon from Algeria, is closely related to *D. lutea* subsp. *lutea* and subsp. *australis* from Corsica. Another common taxon within this alliance is *D. lutea* distributed from the Alps (*D. lutea* subsp. *lutea*) to the Apennine Peninsula and Corsica (*D. lutea* subsp. *australis*). Whilst *D. lutea* subsp. *australis* from Corsica has close affinities to *D. atlantica* from Algeria, *D. lutea* subsp. *australis* from the southern Apennine Peninsula shows clear relationships to *D. subalpina* from Morocco. Consequently *D. lutea* subsp. *australis* is polyphyletic, which means that the taxon from Corsica is not *D. lutea* subsp. *australis* and likewise the taxon from Tuscany should not be placed within *D. lutea*. In this respect one can assume that the ancestors of *D. lutea* subsp. *australis* (Tuscany) and *D. subalpina* on one hand and *D. lutea* subsp. *lutea*, *D. lutea* subsp. *australis* (Corsica) and *D. atlantica* on the other hand occurred sympatrically in northern Africa and the Apennine Peninsula during the Pleistocene. This assumption is

based on palaeogeological data (Lang 1994, Cardona and Contandriopoulos 1979) which support a connection between North Africa and the European mainland 11500–12000 BP. With the breakdown of this landbridge taxa of both lineages were separated and survived as local endemics in northern Africa as well as on the Apennine Peninsula. Some of these rare taxa (Melzheimer pers. comm., Luckner and Wichtl 2000), described as *D. subalpina* var. *cedretorum* (Web.) Ivan., var. *mesatlantica* (Maire) Ivan. and var. *transiens* (Maire) Ivan. occurring in the Atlas mountains (not included in this study) are currently under examination.

Within clade I an ecological separation of taxa can be observed. Members of expanded “*Macranthae*” (*D. grandiflora*, *D. davisiana*, *D. ciliata*; subclade B) are predominantly restricted to the continental parts of Europe whereas representatives of sect. *Digitalis* (*D. purpurea*, *D. thapsi*, *D. mariana*, *D. minor*, subclade A) according to Hinz (1990b) mainly occur in areas with a climate ranging from oceanic-suboceanic to typical mediterranean. This ecological split finds an equivalent in a clear geographical east-west division when comparing the distribution of the most widespread taxa *D. grandiflora* and *D. purpurea* subsp. *purpurea* (Fig. 4, upper map).

Within clade II a polytomy is indicated (subclade D, Fig. 1) with *D. parviflora* as a separate lineage. This local endemic, occurring in northern Spain (Cantabrian Mountains; Sierra del Moncayo), was assigned to sect. *Tubiflorae* subsect. *Obtusisepalae* by Werner (1965) (Fig. 3). Floral morphology and chorological data imply that affinities to *D. subalpina* or to sect. *Globiflorae* are possible (Werner 1961). This is also supported by the molecular data placing all these taxa in one subclade (subclade D). However *D. parviflora* is shown in a polytomy to the branch containing *D. subalpina* and to sect. *Globiflorae*, so a decision between both alternatives for the affinities of *D. parviflora* cannot be made. Due to its isolated position in morphological and geographical respect the taxon in our opinion could be placed in a section of its own,

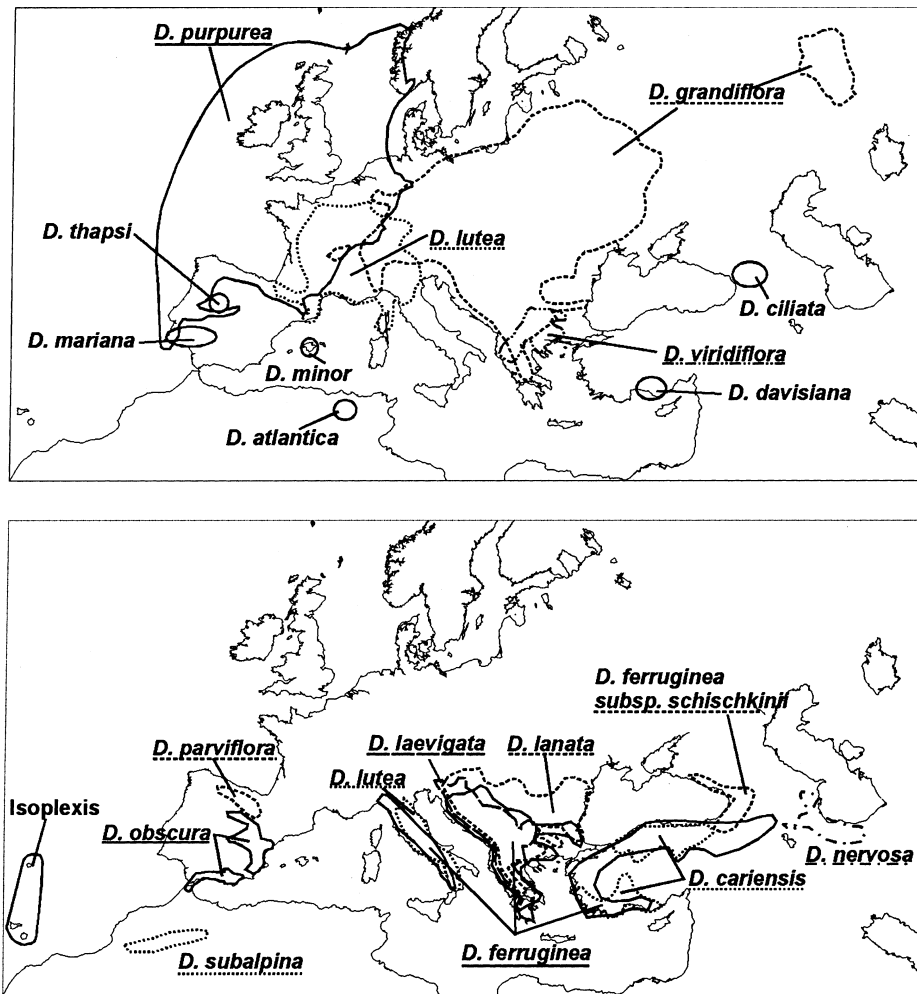


Fig. 4. Schematic distribution pattern of the genus *Digitalis* according to Werner (1965) and Heywood (1972). Biogeographical diversification of clade I (upper map) and clade II (lower map) are compared with tree topology of combined ITS/*trnL*-F analysis. Clade I includes all taxa with an extended distribution in central and western Europe as well as some endemics in the eastern Mediterranean. Clade II is built by *Isoplexis* and taxa of *Digitalis* which have a wide distribution in the southern and eastern Mediterranean as well as by some relicts in the western part of the region

here informally named “*Parviflorae*” (Fig. 3). The position as terminal taxon within subclade D indicates that the isolated position probably could also be inferred from molecular data.

A major group of subclade D (Fig. 1) is composed of sect. *Frutescentes* (*D. obscura*) and taxa of sect. *Tubiflorae* (*D. subalpina* and allies). Similarities between *D. obscura* and *D. subalpina* in growth- and flower morphology already recognized by Werner (1965) are corroborated here once more by the molecular

phylogeny. In consequence either sect. *Frutescentes* could be redefined or in respect to low support of this branch, the *D. subalpina* group could be placed within a section of its own, here informally named “*Subalpinae*” (Fig. 3) while sect. *Frutescentes* could remain unchanged. *Digitalis obscura*, representing sect. *Frutescentes* and placed here in the western group, has a limited distribution in southern Spain (subsp. *obscura*) and the Reef-Atlas of Morocco (subsp. *laciniata*). Werner (1965)

regarded these taxa as the most primitive within the genus *Digitalis* because of their shrubby habit as well as their corolla shape and colour. On the basis of their recent distribution patterns he assumed relationships to the Macaronesian genus *Isoplexis*. However the molecular data provide no evidence for this assumption. The observed differences in both markers may be an indication for a species status of *D. obscura* subsp. *laciniata*, as already proposed by Lindley (1821).

A further distinct group indicated in the phylogeny exclusively consists of taxa of sect. *Globiflorae* which have a main center of diversity in the eastern Mediterranean. When compared to the western alliance of clade II we find a similar topology. It is interesting that *D. ferruginea* subsp. *schischkinii* an endemic taxon occurring in the western Caucasus, is not part of the highly supported monophyly including *D. ferruginea* subsp. *ferruginea*. In this respect *D. ferruginea* could be polyphyletic. The appropriateness of raising this relict subspecies to the rank of a species (*D. schischkinii* Ivan.) needs to be carefully considered. Studies on additional samples and alternative molecular markers are in preparation.

Two further units of taxa are indicated which correspond to subsect. *Hymenosepalae* and subsect. *Blepharosepalae* of sect. *Globiflorae* according to the classification concept of Werner (1965). One lineage includes *D. ferruginea* subsp. *ferruginea*, a widespread taxon on the Balkan and Apennine Peninsulas, *D. laevigata* subsp. *laevigata*, which has a distribution from Dalmatia to the Rhodope Mountains and subsp. *graeca* occurring from the southern Rhodope Mountains to the Peloponnese. A further taxon of this subsect. *Hymenosepalae* is *D. nervosa*, which has a limited and disjunct distribution on the southern side of the eastern Caucasus and in the Elbrus mountains. The affinity of this taxon to *D. laevigata* subsp. *graeca* is unclear, but otherwise the status within the group is confirmed not only by molecular data but also from morphological features. A second lineage within sect. *Globiflorae* is composed of *D.*

cariensis subsp. *trojana*, an endemic taxon from the region adjacent to the Gulf of Edremit in NW Anatolia (Werner 1961), *D. lanata* subsp. *lanata*, which has an extended area on the Balkan Peninsula and subsp. *leucophaea*, with a local distribution from Mount Athos to the island of Thassos in Greece. Sequences of these three taxa assigned to subsect. *Blepharosepalae* by Werner (1965) are identical in both markers. Comprising these findings, sect. *Globiflorae* is confirmed as a natural taxon (Fig. 3). Though subsect. *Blepharosepalae* is monophyletic, the subsectional division needs further examination including all subspecies of *D. cariensis*. Regarding the distribution pattern of the taxa united in subclade D an east-west division parallel to that of clade I can be observed (see Fig. 4 lower map).

Based on our combined ITS/*trnL-F* analysis (Fig. 3) the genus *Digitalis* is a paraphyletic assemblage of taxa with different evolutionary histories. Phylogenetic analyses give evidence for an early split within the genus *Digitalis* and a further sympatric diversification of two lineages in the Mediterranean. Taking in account the distribution of all taxa (as inferred from Werner 1961 and Meusel et al. 1978) one could state that within each of these lineages a western and an eastern group evolved. Within clade I sect. *Digitalis* represents the western and the expanded “*Macranthae*” the eastern group (Fig. 4, upper map), while clade II is composed of sect. *Globiflorae* as eastern lineage and sect. *Frutescentes*, “*Parviflorae*” and “*Subalpinae*” along with sect. *Isoplexis* as western groups (Fig. 4, lower map). Each of the lineages (east and west) contains one relict taxon as well as an alliance of several closely related taxa of recent origin.

Origin and evolution of the *Isoplexis* alliance in Macaronesia. In addition to the problems of an infrageneric subdivision of the genus *Digitalis*, the origin and status of the genus *Isoplexis* has caused much debate among taxonomists (Humphries 1979, Perez de Paz and Roca 1982). This genus is restricted to the Macaronesian archipelago and shows some unusual characters which are considered to be

plesiomorphic, indicating an ancient lineage. This is the observed trend to woodiness and a change in reproductive strategy with a tendency towards ornithophily. On the other hand there is evidence from *Echium* (Böhle et al. 1996), *Sonchus* (Francisco-Ortega et al. 1996) or *Argyranthemum* (Francisco-Ortega et al. 1997) that the distinctive features of endemic taxa of the Canary Islands have originated subsequent to long distance dispersal under the selective pressure of insular environments in a relatively short period.

Although the genus *Isoplexis* has been regarded as a relict taxon which is morphologically and geographically distinct from *Digitalis*, our molecular analysis gives no evidence for this assumption. *Isoplexis*, indigenous to Macaronesia, is a well-supported monophyletic group (subclade C, Fig. 1) which is not indicated in a basal position but unambiguously placed as sister to subclade D of *Digitalis* clade II. This result is also supported by distance analysis (data not shown) where *Isoplexis* is more similar to subclade D than to clade I. Consequently, *Digitalis* sensu Werner (1965) is paraphyletic a fact already mentioned by Carvalho and Culham (1997, 1998). To avoid paraphyly *Isoplexis* should be reduced to sectional rank within *Digitalis* (see Fig. 3) as postulated by Lindley (1821).

Within the *Isoplexis* group *I. sceptrum* from Madeira is the most distant taxon and sister to an unresolved clade including *I. canariensis*, *I. chalcantha* and *I. isabelliana* from the Canary Islands. Recently the genetic relationships and interspecific variation of *Isoplexis* taxa have been analyzed by the application of RAPD markers (Schaller 1998). The species relationships revealed by this study were consistent with our ITS/*trnL*-F phylogeny indicating the isolated position of *I. sceptrum*. Furthermore *I. isabelliana* and *I. chalcantha*, both endemic to Gran Canaria, show a high degree of relatedness and are sister to *I. canariensis* (the most widespread taxon in the Canary Islands occurring on Tenerife and La Palma). The extremely low sequence variation among ITS and high

congruence in RAPD banding patterns between *I. isabelliana* and *I. chalcantha* is also expressed in similarities of cardenolid composition and morphology. According to Luckner and Wichtl (2000) Werner did not regard the differences sufficient to separate these two species. The distinct and isolated position of *I. sceptrum* indicated in the phylogenetic cladogram is also shown on the phytochemical level as this taxon seems to be generally deficient of cardenolids, contrary to other taxa where specific compounds (glycosides of Uzarigenin, Xysmalogenin and Canarigenin) have been identified (Luckner and Wichtl 2000) in addition to other cardenolids common throughout the genus *Digitalis*. If one regards *I. sceptrum* as an ancient taxon of a lineage, separated from mainland *Digitalis* before the cardenolid biosynthesis had been established, then a parallel development of these complex compounds must be postulated. This hypothesis can be rejected in view of the nested position of *Isoplexis* within *Digitalis* and from a chemotaxonomical point of view as we find specific polysaccharides (Digitoxose, Digitalose), identical cardenolides and enzymes of the cardenolid-biosynthesis in both genera.

The growth form of *Isoplexis* as candelabra shrubs often has been cited as a plesiomorphic character (see above). However, regarding the outgroup taxa and the genus *Digitalis* as a whole all species show a trend towards shrubyness (at least woody at the base). Combined with the findings of our molecular analysis it is likely that the habit of *Isoplexis* as an overall woody candelabrous shrub developed under the climatic conditions on the Macaronesian Islands (Carlquist 1974) and has been derived from more or less herbal ancestors, as postulated e.g. for *Echium* in Böhle et al. (1996).

The molecular data provide convincing support for the assumption that island species of *Isoplexis* are derived descendants of continental ancestors. The question whether the colonization of Madeira or the Canary Islands took place first and whether one has been reached via long distance dispersal from the other (as postulated by Carvalho and Culham

1997) or two independent colonization events took place remains obscure.

Origin of ornithophily in *Isoplexis*. All taxa of *Isoplexis* show flower features characteristic for bird pollination (Vogel et al. 1984, Oleson 1985). These are contrasting yellow-orange corollas, flowers without nectar guides, high amount of nectar production, stamens exceeding the corolla tube and reaching out in the upper lip, reduction of lower lip of corolla and thus lack of a landing platform for insect pollinators (Oleson 1985).

There are speculations that taxa with an ornithophilous pollination syndrome are elements of an ancient flora and that true nectar-feeding birds became extinct in Macaronesia (Baez 1992). As it is rather unlikely that the acquired adaptations survived without pollinators for such a long time a stabilizing evolutionary effect must be postulated, achieved by originally non nectarine Passerines that are part of the recent Canary Island avifauna. As described in Vogel et al. (1984) sunbirds (Nectariniidae) nowadays occur from southern Africa northwards up to Near East (Israel). Consequently the assumed occurrence of these birds in NW Africa (Morocco) before the extinction of the evergreen subtropical vegetation in late Miocene/Pliocene is likely and in accordance with biogeographical data (Axelrod 1975). In the Canary Islands different bird species have been reported to be attracted by ornithophilous flowers. Especially *Phylloscopus canariensis* (formerly *Phylloscopus collybita* subsp. *canariensis*) has been observed in the field pollinating *Isoplexis canariensis* and other species (Vogel et al. 1984, Oleson 1985). To a lesser extent also *Sylvia melanocephala* and *S. atricapilla* act as pollinators (Vogel et al. 1984, Oleson 1985), while other species like *Serinus canariensis* seem to exploit flowers illegally without reported pollination. All these birds are elements of the palaeoartic avifauna with no ornithophilous plants in their original (mainland) habitats. The colonization of the Canary Islands by these species probably took place during the Pleistocene and the discovery of nectar as a food source is supposed to be

dated after this event. Though Mühlbauer et al. (2001) postulated a predominantly autogamous strategy for *Isoplexis sceptrum*, bird pollination still seems to play an important rôle for this species (Olesen and Valido 2003).

Regarding the paraphyly of *Digitalis* indicated in our molecular analysis different evolutionary scenarios on the origin of ornithophily in *Isoplexis* could be inferred.

The most parsimonious assumption would be to consider bee pollination (mostly bumblebees of the genus *Bombus*; Knuth 1899) as the most ancestral syndrome in *Digitalis* for it is common in taxa of clade I and subclade D (Fig. 1). Thus bird pollination could have evolved on the Macaronesian Islands de novo or in mainland ancestors of the *Isoplexis* lineage exclusively.

Based on the relationships between *Isoplexis* and the North African/Iberian endemic *Digitalis obscura* a further assumption could be made. Some of the features characteristic for *Isoplexis* seem to be conserved in the latter species, in detail: contrasting orange-yellow corollas and a shrubby habit (though not as distinct as in *Isoplexis*). Regarding *D. obscura* as a relict taxon clade II could also have evolved from ornithophilous mid Tertiary elements of the laurel forests. Remnants of this ancient flora could have spread to the palaeoartic regions and under the new climatic conditions have lost the ornithophilous syndrome which is still present in Macaronesian *Isoplexis*. Consequently bee pollination of recent *Digitalis* species could be regarded as polyphyletic. However taking into account the pollination syndromes of closer relatives of *Digitalis* it is not necessary to state bird pollination as the plesiomorphic character state for the whole genus *Digitalis*.

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