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Using progesterone 5β-reductase, a gene encoding a key enzyme in the cardenolide biosynthesis, to infer the phylogeny of the genus *Digitalis*

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Summary. The progesterone 5β-reductase (5β-POR), a key enzyme in the cardenolide biosynthesis, was sequenced for 21 species of *Digitalis* and *Isoplexis* to infer phylogenetic and biogeographic relationships. This new secondary metabolism molecular marker was compared to the previously applied nuclear ITS and plastid *trn*L-F sequences. The results from separate analyses show high congruence within the genus *Digitalis* and support the conclusion that all species of *Isoplexis* have a common origin and are embedded in *Digitalis*. The genus *Isoplexis* therefore should be reduced to sectional rank within the genus *Digitalis*. The sequence analyses give further evidence that additional sequence data increase support for relationships. It demonstrates that poorly supported relationships in smaller data sets may lead to erroneous conclusions about the evolution of the investigated taxa.

Keywords: Progesterone 5β-reductase (5β-POR); cardenolide biosynthesis; *Digitalis*; *Isoplexis*; phylogeny

Introduction

Plants produce an amazing diversity of low molecular weight compounds. The majority of these compounds belong to "secondary" metabolites that are not essential for the basic metabolism of a plant and whose precise function in the natural environment, however, remains often unclear. The term "secondary compounds" refers mainly to chemicals whose biosynthesis is restricted to selected plant groups and consequently these are of chemo-taxonomical interest. The ability to synthesize specific secondary metabolites has evolved in different plant lineages when such metabolites addressed specific needs. Cardenolide biosynthesis for example has proven useful to ward off pathogens and herbivores of all kinds and has been established several times. The synthesis of cardenolides and their occurrence in general is widely distributed across different orders of angiosperms including

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the genera *Digitalis* and *Isoplexis* (Lamiales, Plantaginaceae). Figure 1 shows the distribution of cardenolides within the Angiosperms (APG 2003). Nevertheless, *Digitalis* plants are still frequently used as raw material and the major source for the isolation of cardenolides applied in human medicine. Although the pathways producing most secondary compounds in plants have not yet been elucidated in detail, it is clear that there are possibly thousands of different enzymes involved (Pichersky and Gang 2000). However, in most cases investigated so far, the enzymes in plant secondary metabolism are specific for a given substrate and produce a single product or group of products. *Digitalis* cardenolides are a good example and valuable drugs in the medication of patients suffering from cardiac insufficiency. The *Digitalis* cardenolides are characterized by a steroid nucleus with rings connected *cis–trans–cis*, which has a 14β-hydroxy group and an unsaturated five-member lactone ring substituted at $C_{17}\beta$. There is a sugar side chain attached at position three in cardenolide glycosides. Through studies using radio-labeled precursors, the putative biosynthetic pathway leading to the cardenolides is basically deduced. Several reviews on cardenolide biosynthesis in foxglove have been published (Kreis et al. 1998, Luckner and Wichtl 2000). In recent years, taking cholesterol as the starting point, more than 20 enzymes which probably affect the formation of cardenolides have been described, identified and characterized (Kreis et al. 1998, Luckner and Wichtl 2000, Herl et al. 2006a). However, molecular data are available only for a few cardenolide biosynthesis specific enzymes like cardenolid-16′-O-glucohydrolase (Framm et al. 2000), lantoside-15′-O-acetylesterase (Kandzia et al. 1998), Δ^5-3β–hydroxysteroid dehydrogenase (Finsterbusch et al. 1999, Lindemann et al. 2000, Herl et al. 2007a). The enzymatic characterization of progesterone 5β-reductase (5β-POR) was reported by Gärtner et al. (1994) and the reaction scheme is shown in Fig. 2. Meanwhile it was possible to clone the corresponding gene (Acc.-Nr. AY585867; Herl et al. 2006a,b). Southern blot analysis revealed that 5β-POR of *D. lanata* is a low copy number gene (Herl et al. 2006a). Similar studies have been conducted for the 3β-hydroxysteroid reductase (3β-HSD) enzyme, followed by the cloning of the respective gene from *D. lanata* by Lindemann et al. 2000 and Herl et al. 2007 (Acc.-Nr. AY844960). Taken into account that both enzymes are closely related to each other and prerequisites for the cardenolide formation in *Digitalis* they serve as good candidates for the investigation of the biosynthetic pathway.

Digitalis and *Isoplexis* traditionally have been placed in the family Scrophulariaceae order Lamiales (see Fig. 1). Recent analyses by Olmstead et al. (2001), Oxelman et al. (2005) and Albach et al. (2005) have established the newly circumscribed Plantaginaceae, in which *Digitalis* and many related genera form a clade with *Plantago* which is well separated from *Scrophularia*. Albach et al. (2005) demonstrated that *Digitalis* is sister to *Erinus* and those two form the sister to the larger clade of *Plantago*, *Aragoa* and *Veroniceae*. Systematics in the genus *Digitalis* (including *Isoplexis*) date back to Linné (1753), although the most detailed and broadly accepted taxonomic treatment was published by Werner (1965), for review see Luckner and Wichtl (2000). Werner (1965) accepted 19 species of *Digitalis* and 3 species of *Isoplexis*. His concept was later slightly modified by additional studies (Hinz 1990) and expanded by the discovery of the new species *I. chalcantha* (Sventenius and O'Shanahan 1969).

Based on a molecular phylogenetic investigation of the genera *Digitalis* and *Isoplexis* using ITS-and *trn*L-F sequences (Bräuchler et al. 2004) it was shown that *Isoplexis* is nested within *Digitalis*. Phylogenetic trees derived from separate analyses were highly congruent. The combined analysis revealed two major lineages, which mark an early split in the genus *Digitalis*. The results further provide evidence that all species of the genus *Isoplexis* have a common origin and are embedded in one of the major lineages of *Digitalis*. It was suggested that *Isoplexis* should be reduced to sectional rank. However, support for many major relationships was weak and, therefore, many conclusions could only be discussed with caution. We investigated the progesterone 5β-reductases (5β-POR) from *Digitalis*, a key

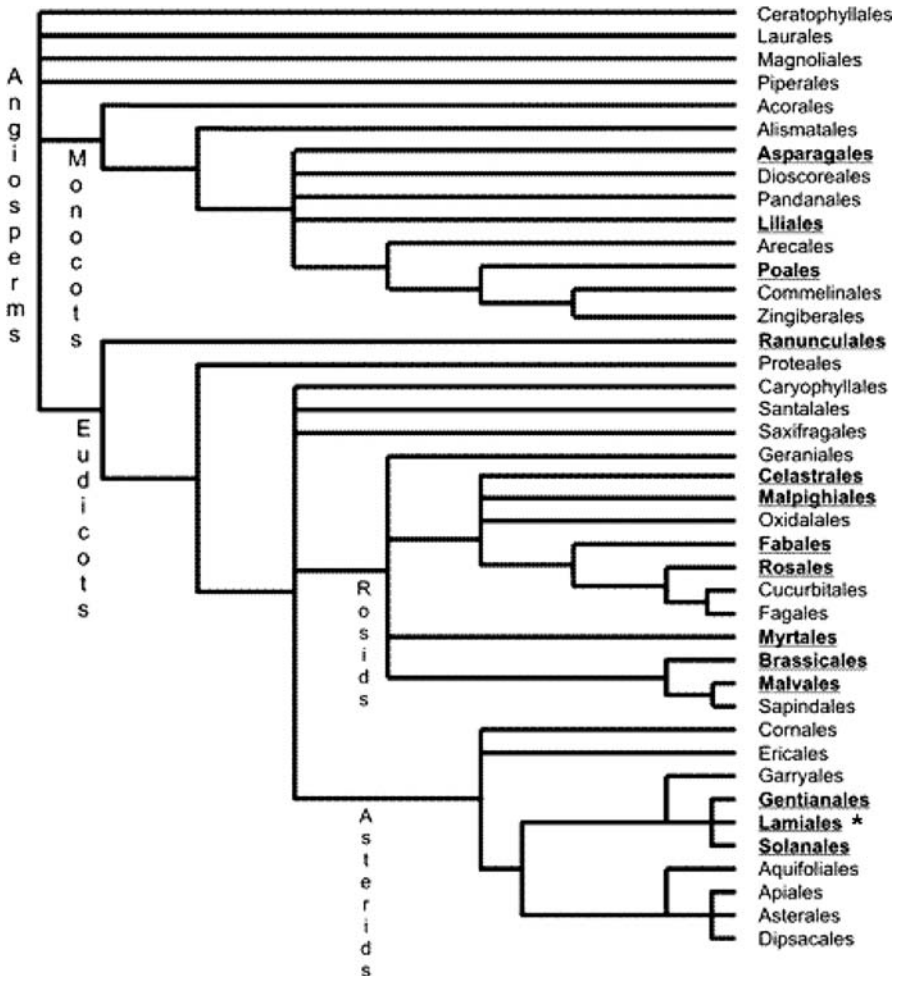

Fig. 1. Distribution of cardenolides in Angiosperms (bold underlined). *Asterisk* indicates the position of the genera *Digitalis* and *Isoplexis*

enzyme in secondary metabolite biosynthesis leading to the 5β-type of the cardenolide structure typically for all *Digitalis* plants (Herl et al. 2006a). Our main aims were: (1) to assess the 5β-POR gene sequences from *Digitalis* and *Isoplexis* species for phylogenetic analysis and (2) to infer and verify the phylogenetic relationships between the genera and sections (3) to test previous systematic treatments by several authors.

Materials and methods

Plant material. Seeds and plant material of *Digitalis* were obtained from the Genbank of the Institute for Plant Genetics and Research on Cultivated Plants in Gatersleben, the Botanical Gardens Erlangen, München, Dresden, Marburg and Halle/S., Germany. *Digitalis minor* specimens were provided by Prof. J. Segura, Valencia, Spain. For the origin of material for other genera of Plantaginaceae see Albach et al. (2005).

DNA/RNA extraction. The plant leaf tissues were ground to a fine powder in liquid nitrogen using a mortar and pestle. Genomic DNA as well as total RNA extraction was carried out with E.Z.N.A.® Plant DNA, resp. RNA Mini Kit (Peqlab, Biotechnologie GmbH, Erlangen). Several of the DNA samples used have been extracted as described earlier (see Table 1; Bräuchler et al. 2004).

PCR amplification. Polymerase chain reaction amplifications of the progesterone 5β-reductase (5β-POR) were performed from total genomic DNA using the primers AAAAAATGAGCTGGTGGTGG (dir) and TGGGCTGGAGCGATCG (rev) as described earlier by Herl et al. (2006a).

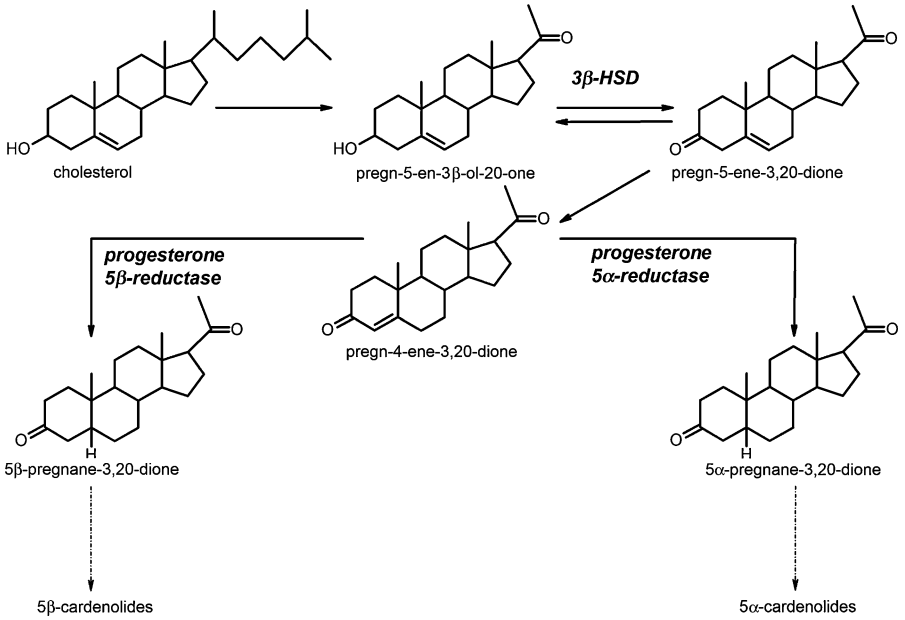

Fig. 2. Early steps in cardenolide genine biosynthesis

Subcloning and sequencing. The PCR bands of the expected size (1250 bp) were extracted with QIAEX II gel Extraction Kit (QIAGEN GmbH, Hilden) and ligated into pCR2.1-TOPO vector for subsequent transformation in *Escherichia coli* strain TOP10 (Invitrogen, Karlsruhe). Transformed cell colonies were selected on ampicillin plates. Plasmid isolation was carried out using the E.Z.N.A.® Plasmid Miniprep Kit (Peqlab GmbH, Erlangen) prior to nucleotide sequence determination (MWG Biotech AG, Martinsried). The sequence was determined from both plasmid ends using forward and reverse primers from the pCR 2.1-TOPO vector. The nucleotide sequences reported in this paper have been submitted to GenBank™ Data Base with accession numbers listed in Table 1.

***In silico* analysis.** After sequencing all data have been analyzed by different software packages (Transeq, Align, European Bioinformatics Institute; Webcutter2, Göteborg University). Data base searching and sequence analysis were performed with a Blast search of the GenBank™ data base. The nucleic acid as well as the translated amino acid sequences were aligned using ClustalW (http://www.ebi.ac.uk/clustalw/index.html).

Phylogenetic analysis. For the analysis we assembled matching data sets of 5β-POR, the ITS- and the *trn*L-F-region (including the trnL intron, trnL 3′-exon and *trn*L-F spacer) for 21 ingroup taxa and 4 outgroups (Table 1). Whereas ITS and the *trn*L-F region contained insertions and deletions, the 5β-POR region did not. Visual inspection of the data matrices revealed five potentially informative gap characters in the ITS region and four in the *trn*L-F-region, which have been scored as absent/present at the end of the data matrix. Data sets were analyzed separately and combined with PAUP* 4.0b10 (Swofford 2002) using heuristic searches with the following settings: ten runs of random taxon addition with ten replicates each using tree bisection reconnection (TBR) were conducted with MulTrees in effect and no tree limit. Due to the lack of an appropriate outgroup for the 5β-POR data set trees were rooted between sect. *Digitalis* and all other species according to results of the ITS-analysis (see below). Parsimony bootstrap percentages were assessed using 1000 replicates with a maximum of 100 trees per replicate; all other conditions are the same as in parsimony analyses (Felsenstein 1985).

Tests of incongruence. Additionally, the Templeton test (Templeton 1983) as implemented in PAUP*4.0b10 (nonparametric pairwise test) was used to compare most parsimonious trees from an analysis of one separate dataset with those from the other two and with those from constraint analyses. For constraint analyses, eight specific relationships found in one analysis but not in the others were used as constraints in eight separate analyses for each of the three DNA regions with the same search strategy as the unconstrained analyses but only one run of random taxon addition (10 replicates). The eight constraints

Table 1. List of the studied taxa with their GenBank accession numbers (5β-POR; ITS, *trn*L-F) and with source/voucher data for 5β-POR sequence

Taxon	Acc.-Nr. ***5β-POR***	Acc.- Nr. **ITS**	Acc.-Nr. ***trn*L-F**	Source/voucher
Digitalis				
D. atlantica Pomel	ND	AY591263	AY591297	*HMelz 23.06.1989*
D. cariensis Boiss. ex Benth.	DQ213016	AY591282	AY591316	*HAL 52609*
D. ciliata Trautv.	DQ213019	AY591264	AY591298	*HAL 1501*
D. davisiana Heywood	DQ213020	AY591267	AY591301	*HAL 716*
D. ferruginea L.	AY738711	AY591279	AY591313	*GG*, *DIGI68/83*
D. grandiflora Mill.	AY585865	AY591261	AY591295	*HAL 73298*
D. laevigata Waldst. & Kit.	DQ213017	AY591281	AY591315	*HAL 65397*
D. lanata Ehrh.	AY585867	AY591285	AY591319	*HAL 74686*
D. lutea L.	DQ213021	AY591266	AY591300	*M*, *France*, *26.07.1990*, de Retz 89971
D. mariana Boiss.	AY738710	AY591259	AY591293	*GG*, *DIGI92/96*
D. minor L. (*D. dubia* sensu Werner (1965)	DQ499643	AY591255	AY591289	*BGV*, *VAL141486*
D. nervosa Steud. & Hochst. ex Benth.	DQ263619	AY591283	AY591317	*HAL 69806*
D. obscura L.	AJ555127	AY591273	AY591307	*HAL 51442*
D. parviflora Jacq.	AY585866	AY591286	AY591320	*HAL 287*
D. purpurea L.	AY585868	AY591257	AY591291	*HAL 79179*
D. subalpina Braun-Blanq.	AY750898	AY591275	AY591309	*HMelz 25.06.1989*
D. thapsi L.	AY738712	AY591256	AY591290	*HAL 51444*
D. viridiflora Lindl.	DQ213018	AY591262	AY591296	*HAL 73433*
Isoplexis (Digitalis)				
D. isabelliana (Webb & Berthel.) Lindinger	DQ218317	AY591270	AY591304	*HAL 178*
D. canariensis Loudon	DQ218315	AY591271	AY591305	*M*, *HB 01.11.2000*
D. chalcantha (Svent. & O'Shanahan) Albach, Bräuchler & Heubl	DQ218316	AY591269	AY591303	*HB*, *Gran Canaria, Bot. Garden Tafira Alta*, *2001*, *Dittrich*
D. sceptrum Loudon	DQ218318	AY591268	AY591302	*M, Madeira*, *10.09.1986*, *Hertel 33616*
Globularia salicina Lam.	–	AF313039	AF513358	*Chase 2547*, *K*
Plantago uniflora L.	–	AY101885	AY101940	*Chase 2798*, *K*
Veronica montana L.	–	AF313014	AF486388	*Albach 151*, *WU*
Wulfenia carinthiaca Jacq.	–	AF313025	AF486409	*Albach 74*, *WU*
Arabidopsis thaliana L.	EF579963			*Welss 101*, *ER*

See Bräuchler et al. (2004) and Albach et al. (2005) for vouchers of the other sequences
HB Herbarium Bräuchler, München; *HMelz* Herbarium Prof. V. Melzheimer; *BGV* Bot. Garden Valencia, Spain; *GG* Genbank Gatersleben; *M* Bot. Garten München; *HAL* Bot. Garten Halle; *ER* Bot. Garten Erlangen; *WU* Vienna university; *K* Royal Botanic Gardens Kew

were 1. *D. ciliata* sister to the rest of sect. *Macranthae*, 2. *D. lutea* sister to the rest of sect. *Macranthae*, 3. *D. viridiflora* sister to the rest of sect. *Macranthae*, 4. Sect. *Isoplexis* sister to the species not belonging to sect. *Digitalis* + *Macranthae*, 5. *D. parviflora* sister to those species, 6. *D. parviflora* sister to *D. subalpina*, 7. *D. subalpina* sister to *D. obscura*, 8. *D. obscura* sister to sect. *Isoplexis*.

Results and discussion

When the nucleotide sequence of the cDNAs were analyzed *in silico* high similarity was found for all 5β-POR available in the GenBank™ Database. Species for the analyses were chosen according to Werner (1965) as reported earlier (Bräuchler et al. 2004). Only the respective 5β-POR of *D. atlantica* and some taxa recognized below the rank of species are missing. Herbarium material (kindly provided by Prof. V. Melzheimer, Marburg) did not provide DNA suitable for PCR amplification of 5β-POR. Amplification of 5β-POR was unsuccessful in seven other genera of Plantaginaceae (*Antirrhinum molle*, *Erinus alpinus*, *Gratiola officinalis*, *Penstemon whippleanum*, *Picrorhiza kurrooa*, *Veronica montana*, *Wulfenia carinthiaca*). The reading frame of the 5β-POR gene is about 1,170 nucleotides long corresponding to about 389 amino acids. The aligned sequences had 1,170 positions, of which about 1060 (90%) were constant, 110 (10%) were variable, including 45 parsimony informative characters. In 45% of the 110 variable positions the nucleotide changes led to an amino acid substitution in the protein sequence. The G + C content is about 47.8% (Table 2).

Taxonomic treatments of the genus *Digitalis* (including *Isoplexis*) based on morphology have been described earlier by Ivanina (1955), Werner (1965), Bocquet and Zerbst (1974), Hinz (1990), for review see Luckner and Wichtl (2000). First results using molecular markers have already shown the close relationship of *Digitalis* with *Plantago* and *Veronica* within the Plantaginaceae "Scroph II" Veronicaceae (Olmstead and Reeves 1995; Olmstead et al. 2001; Albach et al. 2001, 2005). Most molecular systematic approaches applied to *Digitalis* have been limited to a few species only (Perez de Paz and Roca 1982; Carvalho and Culham 1997, 1998; Schaller 1998; Nebauer et al. 2000; Sales et al. 2001; Gavidia et al. 1996, 2002a, b, 2007). Bräuchler et al. (2004) have been the first to investigate all species of the genus using nuclear internal transcribed spacer and plastid *trn*L-F sequences and presented a phylogenetic tree of all species of the genus. Their results showed the integration of the *Isoplexis* spp. within *Digitalis* as a monophyletic group. Several biochemical parameters have been applied to verify the taxonomy of the genus based on morphological characters. Especially the chemotaxonomical parameters nicely fit to the first established evolutionary system of the genus (based on Werner (1965). Earlier investigation of different *Digitalis* species concerned the cardenolide pattern. Luckner and Wichtl (2000) compiled the data concerning the chemical pattern (Digoxigeninderivates, Gitoxigeninderivates, Tetrasaccharideglycosides, etc.) and the content of cardenolides from several *Digitalis* species. They found that the cardenolide pattern and the amount of cardenolides of, e.g., *Digitalis obscura (Frutescens)* and *Digitalis ferruginea (Globiflorae)* are more similar to each other than to other species from *Macranthae* or *Digitalis*. The deduced schema showed that some kind of taxonomy of the species (into the different

Table 2. Sequence characteristics of the data sets used for phylogenetic analysis and statistics of the most parsimonious trees

	ITS	*trn*L-F	5β-POR
Number of characters	663	912	1170
Parsimony informative characters	179 (90 within *Digitalis*)	53 (10 within *Digitalis*)	45
Coded indel characters	5	4	–
GC content	50.4%	35.4%	47.8%
Number of most parsimonious trees	4	1	72
Length of most parsimonious trees	568	229	144
CI (scoring informative characters only)	0.73	0.92	0.72
RI	0.75	0.88	0.87

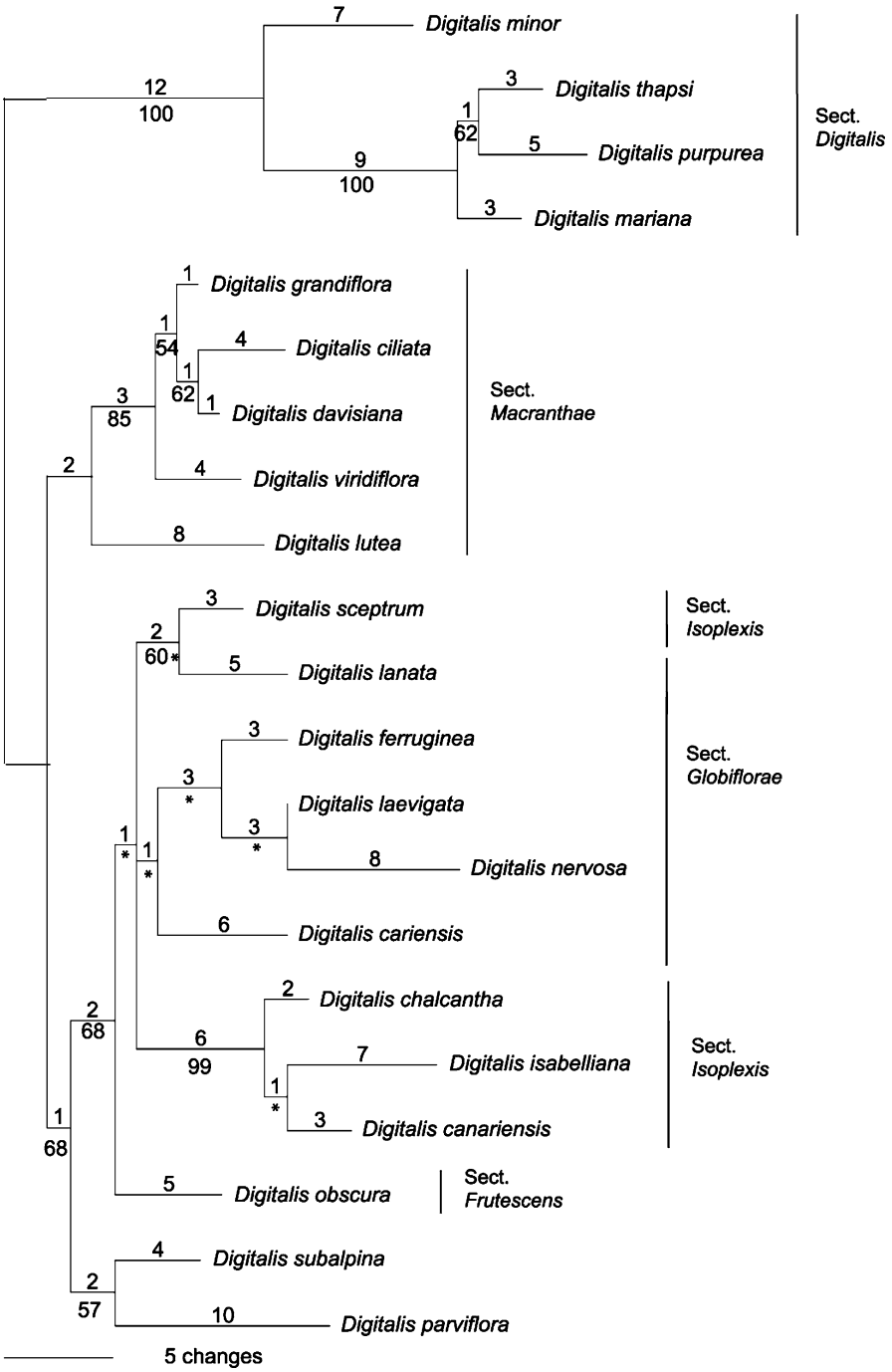

Fig. 3. One of 72 most parsimonious trees of the analysis of the 5β-POR-data set. Branches not present in all most parsimonious trees are marked with an *asterisk* below the branch. Numbers above the branches indicate branch lengths, those below the branch bootstrap percentage

clades as by Werner 1965) could also be created on the basis of these chemo-taxonomical parameters.

Phylogenetic reconstruction based on the gene sequence alignment for 5β-POR from *Digitalis* revealed 72 most parsimonious trees

supporting earlier taxonomic treatment to some extent (Fig. 3). The analysis of the 5β-POR region reveals the same larger clades in *Digitalis* found by Bräuchler et al. (2004), although not all most parsimonious trees show sect. *Macranthae* or *Isoplexis* as monophyletic. In some trees *Isoplexis sceptrum* is dissociated from other *Isoplexis* taxa and sister to *D. lanata* (60% BS). However, in all other most parsimonious trees *D. lanata* is sister to *D. cariensis* and sect. *Isoplexis* is monophyletic. Furthermore, *D. subalpina* and *D. parviflora* are sister species (57% BS).

5β-POR sequences are highly conserved not only within the genus *Digitalis* but may be obtained also in other species, e.g. *Arabidopsis*

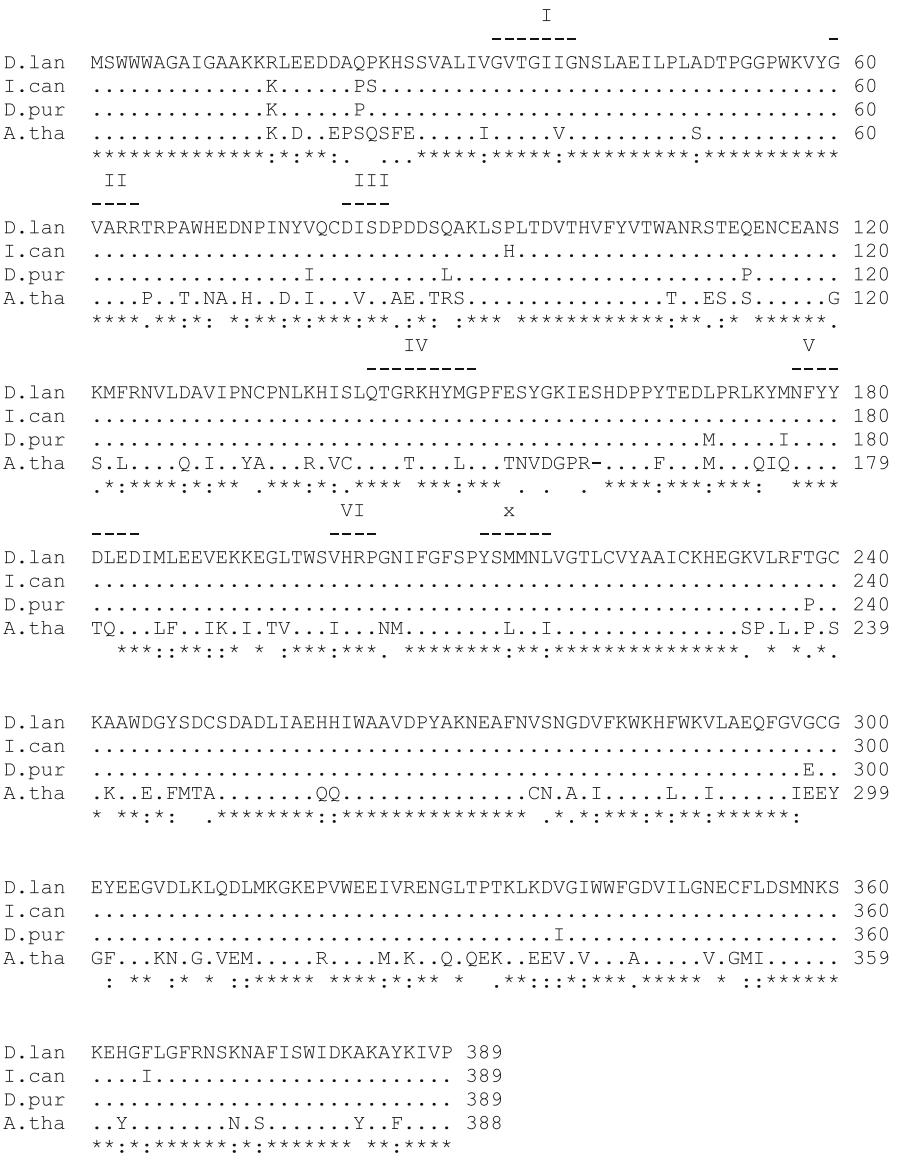

Fig. 4. Alignment of the deduced and functional 5β-POR proteins from *Isoplexis canariensis* (I.can; DQ218315), *Digitalis lanata* (D.lan; AY585867), *Digitalis purpurea* (D.pur; AJ310673) and *Arabidopsis thaliana* (A.tha; AAL32529.1). The consensus sequence and conserved motifs are indicated by *asterisks*

(Fig. 4). An unknown protein from *Arabidopsis thaliana* (Acc.-Nr. At4g24220/EF579963) shows considerable sequence identity (67%) to 5β-POR of *Digitalis*. However, 5β-POR sequences could not be amplified from other related taxa (see above). Recombinant proteins from cDNA clones of *Digitalis lanata*, *Isoplexis canariensis* and *Arabidopsis thaliana* result in functional 5β-POR enzymes (Herl et al. 2006a, b; 2007 unpublished).

Using the *Arabidopsis* sequence as an outgroup results in a basal split between sect. *Digitalis* and the rest but with little bootstrap support (tree not shown). Information on the individual data sets and the most parsimonious trees found is given in Table 2. The results of the ITS and *trn*L-F analyses (Fig. 5) are essentially identical to a recent analysis (Bräuchler et al. 2004) except for the root in the ITS-analysis located between sect. *Digitalis* and the remaining species (90 and 80% BS, respectively) rather than between sect. *Digitalis* plus sect. *Macranthae* and the rest, due to different choice of outgroup.

The combined data set of all three DNA regions includes 2,745 characters, 277 (145 within *Digitalis*) of these plus nine indel characters are potentially parsimony informative. The 40 most parsimonious trees have 954 steps (Fig. 6; CI = 0.65 scoring only informative characters, RI = 0.78). The analysis retrieves the same three major clades found in almost all separate analyses: sect. *Digitalis*, sect. *Macranthae* and the remaining species. As in the analysis

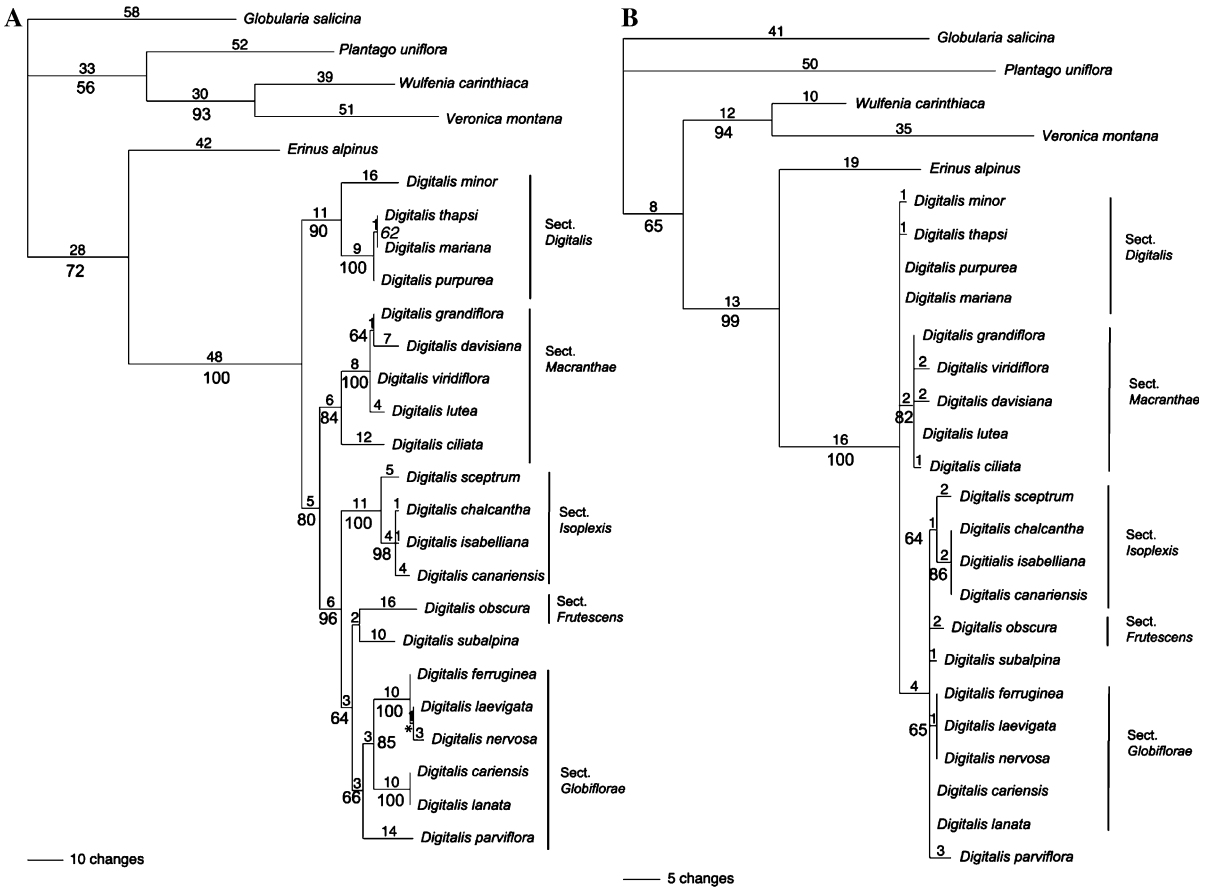

Fig. 5. Most parsimonious trees of the analysis of the ITS- (A) and *trn*L-F-data set (B). For the ITS-analysis one of the four most parsimonious trees is depicted with the branch not present in the strict consensus marked with an *asterisk* under the branch. Numbers above the branches indicate branch lengths, those below the branch bootstrap percentage

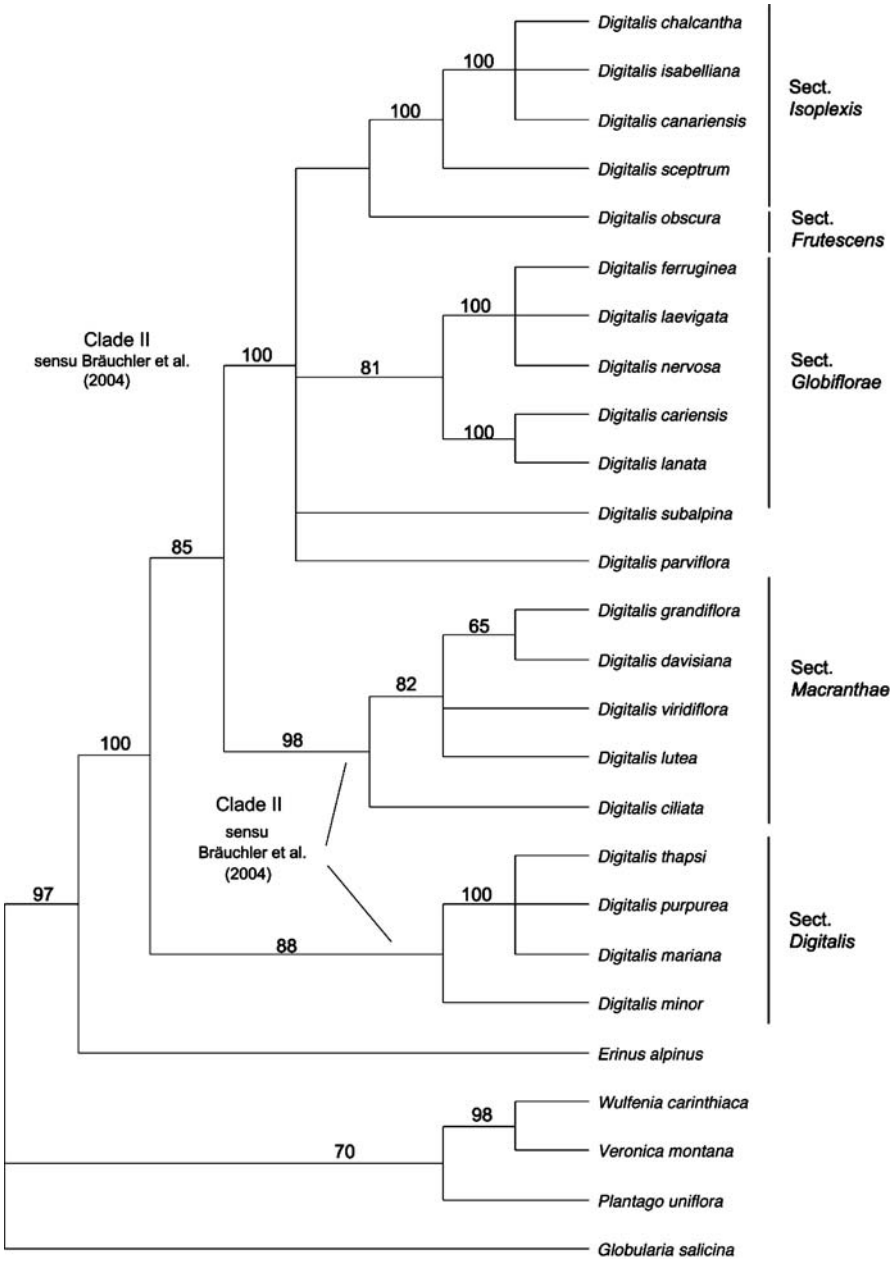

Fig. 6. Strict consensus tree of the parsimony analysis of the combined analyses of all three data sets. Numbers above the branches indicate bootstrap percentage

of ITS, the root is located between sect. *Digitalis* and the remaining species (88 and 85% BS, respectively). Support for sect. *Macranthae* increased in the combined analysis (98% BS), whereas support for relationships within the section decreased. Relationships among the other species remain unclear. Support for sect. *Isoplexis* (100% BS) and sect. *Globiflorae* (81% BS) are high but *D. subalpina* (sister to all these species) and *D. obscura* (sister to sect. *Isoplexis*) occur at positions not found in the separate analyses but also not supported by the bootstrap analysis. The position of *D. parviflora* is unresolved.

The Templeton-test clearly rejects the topology of the *trn*L-F- ($p < 0.001$) and 5β-POR-analysis ($p < 0.001$) for the ITS data set and the *trn*L-F- ($p < 0.001$) and ITS-topology ($p < 0.01$) for the 5β-POR-data set. The tests using the *trn*L-F data set marginally do not reject the other topologies (ITS: $p = 0.16$; 5β-POR: $p = 0.05$–0.16). None of the eight constraints led to the rejection by one of the three DNA regions.

Our results give further evidence that additional sequence data increase support for relationships found previously using less data. It also demonstrates that poorly supported relationships in smaller data sets may lead to erroneous conclusions about the evolution of the investigated taxa. The analysis of the 5β-POR data set separately and combined supports earlier conclusions (Bräuchler et al. 2004) about the division of the genus in sect. *Digitalis*, sect. *Macranthae* and clade II (remaining species) and the further sectional division of *Digitalis*. All sections (as defined by Bräuchler et al. 2004) are monophyletic. However, our study demonstrates that the question of the origin of sect. *Isoplexis* is not resolved. Analyses of ITS sequences (Bräuchler et al. 2004; Fig. 5) support a division of clade II in sect. *Isoplexis* and all other species, and thus support an early origin of the section and the relictual nature of the group on the Canary Islands. However, our combined analysis reveals *D. obscura* as sister to sect. *Isoplexis* (Fig. 6), a relationship supported by morphology (Werner 1965), and both nested within clade II. Considering this position and that *D. obscura* is among the westernmost species of the genus, this implies a more recent origin of sect. *Isoplexis* from western Mediterranean ancestors. A recent origin of insular woodiness on the Canary Islands in sect. *Isoplexis* from herbaceous to subshrubby ancestors is in line with results from other plant taxa (e.g. Böhle et al. 1996, Kim et al. 1996; Moore et al. 2002, Fairfield et al. 2004). Furthermore, the combined analysis demonstrates that relationships within the sections are poorly supported by ITS and *trn*L-F alone. Adding a third dataset and increasing the number of informative sites by about 50% changes the outcome of the analyses considerably, however without increasing support. A good example is the relationship of *D. subalpina* and *D. parviflora*, suggested to be closely related by Werner (1965), but not by ITS and *trn*L-F (Bräuchler et al. 2004; Fig. 5). Both species are sister to each other in the analysis of 5β-POR (Fig. 4), but the two characters supporting this relationship seem not to be enough to lead to this conclusion in the combined analysis (Fig. 6). It demonstrates that poorly supported relationships in smaller data sets may lead to erroneous conclusions about the evolution of the investigated taxa.

One surprising relationship in some of the most parsimonious trees from the 5β-POR analysis and supported by 60% of the bootstrap replicates is the clade comprising *D. sceptrum* and *D. lanata*, thus rendering sect. *Isoplexis* polyphyletic. Visual inspection of the informative characters reveals that lack of enough informative characters rather than anything else is the reason for this result. The 5β-POR data set does not only have about half the number of informative characters as the ITS data set (45 vs 95) but those 45 characters are also unevenly distributed with 15 of them being only variable in sect. *Digitalis*. The relationship of *I. sceptrum* and *D. lanata* is supported by two characters and the same number of characters supports the relationship of *D. lanata* with *D. cariensis* as found in the ITS analysis (Bräuchler et al. 2004; Fig. 5) and the combined analysis (Fig. 6). Furthermore, although four characters uniquely support the branch leading to *D. canariensis*, *D. chalcantha*, and *D. isabelliana*, none supports uniquely sect. *Isoplexis*. Both characters that do support sect. *Isoplexis* also support the relationship of the section with *D. lanata* and *D. cariensis*. Thus, monophyly of sect. *Isoplexis* is not refuted by the 5β-POR data set. Together with the results from the comparison of constraint analyses this demonstrates that character sampling in *Digitalis* has not reached a level, yet, that gives robust phylogenetic hypotheses across the genus.

Only a detailed examination of the presence or absence of particular reactions in related plant species whose true phylogeny is known can allow us to determine which reactions came first. We are attempting to do such an analysis in a

particular case the cardenolide biosynthesis. An important consequence of repeated evolution, in which a new genetic function arises independently but from orthologous or paralogous genes, is that the catalytic function of a newly described gene or protein cannot be assigned solely on its degree of sequence identity to known enzymes, although this is currently common practice (Pichersky and Gang 2000).

In conclusion, this study confirms that the genus *Digitalis* also includes *Isoplexis*. Therefore names under *Isoplexis* should be considered synonyms of those combinations under *Digitalis*. One necessary new combination is made here for *Digitalis chalcantha* (Svent. & O'Shanahan) Albach, Bräuchler & Heubl **comb. nov.** (basionym: *Isoplexis chalcantha* Svent. & O'Shanahan in Ind. Sem., Agron. Investig. Nat. Hispan. Inst. 1968, 47 (1969)). The comparison of the sequences for low-copy genes (e.g., 5β-POR) provides useful new information for the phylogenetic reconstruction of the organismic evolution and provides a useful start for further studies to increase our understanding of the cardenolide biosynthesis and its evolution.

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