Diosgenin Contents and DNA Fingerprint Screening of Various Yam (*Dioscorea* sp.) Genotypes

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Dedicated to Prof. Wilhelm Fleischhacker on the occasion of his 75th anniversary

In addition to the importance of many *Dioscorea* species (yams) as starchy staple food, some representatives are known and still used as a source for the steroidal sapogenin diosgenin, which, besides phytosterols derived from tall-oil, is an important precursor for partial synthesis of steroids for pharmaceutical research and applications. While in edible yams the diosgenin content should be as low as possible, a high yield of the compound is preferable for cultivars which are grown for the extraction of sterols. In the past, miscalculations and insufficiently precise techniques for quantification of diosgenin prevailed.

Therefore we set out to re-evaluate the steroid content of a world collection of *Dioscorea* species, using leaves as sample material. We optimized diosgenin quantification techniques and fingerprinted the whole collection with the DNA amplification fingerprinting (DAF) technique. Total diosgenin contents ranged from 0.04 to 0.93% of dry weight within the collection. Several *Dioscorea* cultivars can be characterized via their DAF fingerprint patterns.

Key words: Dioscorea, Diosgenin Production, DNA Amplification Fingerprinting

Introduction

Yams (*Dioscorea* sp.) are widely distributed. They are economically important tuber and bulbil crops in tropical and subtropical regions worldwide. They have been used as aliment since ages, and 13 out of some 40 cultivars can be regarded as regional or worldwide important food plants (Rehm and Espig, 1984). Apart from the traditional importance as starchy staple food, some *Dioscorea* species are known and used as a source for the steroidal sapogenin diosgenin, a precursor for the synthesis of steroid drugs (Djerassi, 1992; Chu and De Cássia Leone Figueiredo Ribeiro, 2002). In fact, pharmaceutical phytosterols have primarily been derived from tall-oil, a byproduct of wood processing in the cellulose industry (Dias *et al.*, 2002), but second in importance were (and still are) field cultures of *Dioscorea* plants, that produce diosgenin derivatives in leaves and deposit them in their storage tubers. However, field cultures of *Dioscorea* have various limitations as *e.g.* poor seed germination (Forsyth and Van Staden, 1982) and continuous exposure to viral, bacterial and fungal pathogens and nematodes (Ng, 1988) with inevitable yield losses. Moreover, the extensive harvesting of wild plants for pharmaceutical use endangered several *Dioscorea* species in regions such as Mexico and South Africa (Coursey, 1967). Therefore, and rather early in yam research, *in vitro* cell cultures have been established as an alternative to field plantations. The production of diosgenin in such cell cultures, however, was variable (Aminuddin and Chowdhury, 1983; Ćulafić *et al.*, 1999; Kaul and Staba, 1968). Yet the failure of achieving fully chemical synthesis of steroids until now again made *Dioscorea* a very attractive source for steroidal precursors.

While edible *Dioscorea* species should lack exceeding amounts of steroids, a preferably high

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content is of importance for those species which serve as raw material for the isolation of diosgenin derivatives. From the early 1940 on, diosgenin contents of various *Dioscorea* species and several different organs were repeatedly estimated, but the values published by several authors are to be compared with caution and should only partially be considered as contribution to an overall diosgenin screening of the whole genus, since analytical methods as well as the tissue samples varied considerably (Akahori, 1965; Anzaldo, 1956; Barua *et al.*, 1954, 1956; Czikow and Łaptiew, 1983; Marker *et al.*, 1943; Quigley, 1978; Wall *et al.*, 1952a, b, 1954a, b, 1955, 1957). Also, in many cases the techniques were far from advanced. In his book "Yams", Coursey (1967) reported of diosgenin contents of several *Dioscorea* species, which had been estimated by the above-mentioned authors, on a percent basis. However, among others he quoted figures from publications by Marker *et al.* (1943), which were originally reported as $g\,kg^{-1}$, as percent, as well. Thus, those diosgenin values in the book "Yams" adopted from Marker and colleagues are tenfold too high. As this book has been considered a standard for *Dioscorea*-related research since then, the incorrect values have been uncritically adopted by further authors. This combination of rather imprecise techniques, wrong quotations and inherent variability of diosgenin contents led to much confusion and practically a cessation of yam research.

For all of these reasons we considered it essential to establish a collection of as many *Dioscorea* clones *in vivo* as we were able to procure as well as to develop a uniform procedure for sample preparation and diosgenin analysis, which altogether aimed at retrieving reliable informations on the diosgenin content of various *Dioscorea* species and cultivars of several species. To our knowledge, diosgenin screening has never been carried out with a comparably large collection of *Dioscorea* accessions that are all grown under the same conditions in the same greenhouse. In order to monitor the reliability of classification of the accessions we received from several regions worldwide, to correct misclassifications and to examine the potential of genetic distinction between species and cultivars within the same test setup, we set out a DNA fingerprint screening upon our entire collection of *Dioscorea* clones, using the DAF (DNA amplification fingerprinting) procedure.

Materials and Methods

Plant material

The various *Dioscorea* species and cultivars employed are listed in Table I. They were either provided as bulbils, tubers or *in vitro* plantlets. Usually bulbils and tubers were germinated in moist soil, *in vitro* plantlets were transferred to soil and left in a mist chamber for 4 weeks. The emerging, acclimatized plants were grown in the greenhouse under normal day-night regimes prevailing in Central Europe.

Diosgenin quantification

Sample preparation, extraction and hydrolysis were performed as described by Farnleitner (2004), consisting of the following procedures: Lyophilized plant material was ground in a Retsch ZM-100 centrifuge mill (0.5 mm mesh sieve). For each sample, 1.00 g of ground material was suspended in 20 mL water for 24 h at room temperature for enzymatic pre-hydrolysis. For extraction of dioscin and already pre-hydrolyzed diosgenin, 30 mL *iso*-PrOH were added, which resulted in a 60% (v/v) aqueous *iso*-PrOH solution. After estimation of the exact weight, the mixture was heated to 100 °C in a water bath for 3 h and then centrifuged at 3500 rpm for 7 min. The supernatant was weighed again before additional *iso*-PrOH and H_2SO_4 were added in a proportion leading to a final concentration of 2 M H_2SO_4 in 70% (v/v) *iso*-PrOH/30% H_2O. Hydrolysis proceeded for 4 h at 100 °C in a water bath. 3.0 mg progesterone (Sigma Aldrich; purity: 98.45%) as an internal standard were added. The solution was diluted 50:50 (v/v) with distilled H_2O and extracted three times with 30 mL dichloromethane. The collected organic phases were first deacidified with 30 mL and then 60 mL 5% (m/v) aqueous $NaHCO_3$. The organic phase was dehydrated with Na_2SO_4. After filtration, the solvent was evaporated under reduced pressure and the residue was re-dissolved in 4 mL dichloromethane for GC analysis [apparatus: Perkin Elmer Auto System XL gas chromatograph; carrier gas: nitrogen; flow rate: 2 mL min^{-1}; injection: splitless mode, 290 °C; injection volume: 1 μL; column: HP-5 (crosslinked 5% Ph Me Silicone), 50 m × 0.32 mm i. d. × 0.52 μm; temperature program: initial temperature 290 °C, 1 °C min^{-1}, final temperature 310 °C, hold 10 min; detection: FID; quantification was accom-

Table I. An inventory of all *Dioscorea* species and cultivars used in the present study.

Species	Cultivar (subspecies)	Origin (country)
D. alata	7087[a]	University of Frankfurt (Germany)
D. alata	E-1-04	Ventanas, Los Rios (Ecuador)
D. alata	E-1-06	Ventanas, Los Rios (Ecuador)
D. alata	TDa 289	Intl. Institute of Tropical Agriculture, IITA (Nigeria)
D. alata	TDa 1349	Intl. Institute of Tropical Agriculture, IITA (Nigeria)
D. alata	V-1-12	Maracay, Carabobo (Venezuela)
D. alata	V-1-30	Maracay, Carabobo (Venezuela)
D. alata	V-1-34	Maracay, Carabobo (Venezuela)
D. alata	V-1-51	Maracay, Carabobo (Venezuela)
D. alata	V-1-53	Maracay, Carabobo (Venezuela)
D. alata	V-1-55	Maracay, Carabobo (Venezuela)
D. alata	V-1-59	Maracay, Carabobo (Venezuela)
D. alata	V-1-60	Maracay, Carabobo (Venezuela)
D. alata	V-1-61	Maracay, Carabobo (Venezuela)
D. alata	V-1-63	Maracay, Carabobo (Venezuela)
D. alata	V-1-70	Maracay, Carabobo (Venezuela)
D. alata	V-1-87	Maracay, Carabobo (Venezuela)
D. alata	V-1-90	Maracay, Carabobo (Venezuela)
D. alata	V-1-95	Maracay, Carabobo (Venezuela)
D. alata	V-1-99	Maracay, Carabobo (Venezuela)
D. batatas		University of Vienna (Austria)
D. bulbifera	DB 01	Antananarivo (Madagascar)
D. bulbifera	DB 02	University of Frankfurt (Germany)
D. bulbifera	cv. *elongata* DB 06	Nambayufa (Papua New Guinea)
D. bulbifera	cv. *heterophylla* DB 10	Sandimen (Taiwan)
D. bulbifera	cv. *heterophylla* DB 18	Cheng Sen (Thailand)
D. bulbifera	cv. *anthropophagorum* DBET 9307	Majan III (Ethiopia)
D. bulbifera	PM3	University of Frankfurt (Germany)
D. bulbifera	cv. *vera* DB 254	University of Frankfurt (Germany)
D. bulbifera	cv. *vera* DB 268	University of Frankfurt (Germany)
D. caucasica		University of Frankfurt (Germany)
D. cayenensis	blackwiss #12	Manchester (Jamaica)
D. cayenensis	yellow AFU yam	Spanish Town (Jamaica)
D. cayenensis	TDc 95-293	Intl. Institute of Tropical Agriculture, IITA (Nigeria)
D. cayenensis	TDc 95-294	Intl. Institute of Tropical Agriculture, IITA (Nigeria)
D. cayenensis/rotundata	tau yam (intermediate)	Manchester (Jamaica)
D. composita		University of Vienna (Austria)
D. deltoidea	cv. "Bombay"	Zandu Pharmaceutical Works Ltd. (India)
D. deltoidea	cv. "Bayreuth"	University of Bayreuth (Germany)
D. discolor		Botanical Garden Halle (Germany)
D. japonica		University of Frankfurt (Germany)
D. mangenotiana	TDm 2938	Intl. Institute of Tropical Agriculture, IITA (Nigeria)
D. nipponica		Botanical Garden Halle (Germany)
D. pentaphylla		University of Vienna (Austria)
D. sp.		University of Vienna (Austria)
D. reticulata		University of Bayreuth (Germany)
D. rotundata	TDr 608	Intl. Institute of Tropical Agriculture, IITA (Nigeria)
D. rotundata	TDr 747	Intl. Institute of Tropical Agriculture, IITA (Nigeria)
D. rotundata	TDr 3444	Intl. Institute of Tropical Agriculture, IITA (Nigeria)
D. sansibarensis		Botanical Garden Berlin (Germany)
D. vittata		University of Frankfurt (Germany)

[a] The accession received as *D. alata* 7087 was identified as *D. bulbifera* and will subsequently be referred to as *D. bulbifera* DB 7087.

plished by integration of the respective peak areas].

For the identification of diosgenin and the isomere yamogenin, GC-MS analysis was applied [apparatus: Shimazu GC-MS-QP5050; carrier gas: helium; flow rate: 2 mL min^{-1}; injection: splitless mode, 290 °C; injection volume: 1 μL; column: Perkin Elmer Elite SE 54 column (60 m ×

0.25 mm i. d. × 0.25 μm), temperature program: as given above; detection: MS, electronic impact (70 eV), mass range was from 40–60 amu, scan interval was set at 1 s]. Identification was achieved via comparison of the collected mass spectra with those of reference compounds from mass spectral libraries.

DNA extraction and purification

For DNA amplification fingerprinting, intact leaves were harvested, immediately shock-frozen in liquid nitrogen, and subsequently extensively lyophilized. The freeze-dried leaves were then ground in a sterile mortar, and from the resulting powder, total DNA was isolated using a modified CTAB procedure (Weising *et al.*, 1991). Crude DNA preparations were dissolved in an appropriate volume of TE buffer (30 mM Tris-HCl, 2 mM EDTA, pH 7.5).

DNA amplification fingerprinting and electrophoresis

DAF followed the procedure of Caetano-Anollés *et al.* (1991) with the following modifications: PCR was carried out on an Eppendorf Mastercycler thermal cycler using random 10-mer primers (random kits C, X and Y) procured from Roth, Germany. Each 15 mL-PCR master mix contained 1.5 mL of 10 × PCR buffer, 2 mM of $MgCl_2$, 10 mM of dNTP-mix, 0.4 U of Biotherm *Taq* DNA polymerase, 40 pmol of oligonucleotide primer and 1 ng mL^{-1} of template DNA. The DNA was first denatured for 2 min at 95 °C, followed by 40 cycles of 15 s denaturation at 95 °C, 1 min annealing at 35 °C and 2 min elongation at 72 °C, with a final elongation at the same temperature for 2 min. The reaction products were separated on 2% agarose gels, stained with ethidium bromide and visualized by UV light.

Results

Diosgenin content

Although the highest content of diosgenin glycosides throughout a full-grown *Dioscorea* plant resides in the tubers or rhizomes, respectively, where these substances are stored, we decided to carry out diosgenin analyses of leaves. Reasons are, that diosgenin glycoside contents in subterranean organs such as rhizomes or tubers seem to vary with the age of the plant and hence individuals of different age cannot be compared. In addition, some of the plants grown in our greenhouse were so young that they had not yet developed tubers from which an appropriate amount of sample material could have been removed without causing severe damage or, probably, loss of the plant. The total diosgenin content of leaf material after hydrolysis was estimated for all 51 accessions (Table I) via gas chromatography (GC). 1 g of lyophilized leaf material per analysis was extracted with 60% (v/v) 2-propanol and hydrolyzed 4 h in a 2 M H_2SO_4-2-propanol solution. Progesterone was used as an internal standard for GC. Quantification of diosgenin and yamogenin was accomplished by integration of corresponding peak areas on the resulting chromatograms.

As Table II shows, total diosgenin contents (= sum of diosgenin and yamogenin content) of leaves ranged from 0.04 to 0.93% of dry weight. The highest diosgenin value was estimated in *D. rotundata* TDr 608. Among the tested cultivars of this particular species, the lowest value was 0.45%, hence *D. rotundata* is the species with the highest average diosgenin content of our collection. *D. cayenensis* ranks second with diosgenin contents ranging from 0.31 to 0.73%. Statistically, *D. alata* is the species with the lowest diosgenin content, with only 6 cultivars out of 19 exceeding 0.1%.

For correct data analysis, two separate samples of each 1.00 g of lyophilized leaf tissue were extracted and hydrolyzed, and two injections were performed separately for each hydrolysate, thus providing four values per cultivar. The values compiled in Table II represent the arithmetric mean values from the four estimated values of each sample.

DNA amplification fingerprinting

A collection of plants representing 18 species of 51 accessions of the genus *Dioscorea* was established in a temperature-controlled greenhouse under normal day-night conditions prevailing in Central Europe. For DNA fingerprinting, 10–20 g of leaves were harvested, immediately shock-frozen in liquid nitrogen and lyophilized. DNA was extracted from 0.2 g of lyophilized plant material and used for DAF. The band patterns resulting from DAF analysis of selected representatives, one of each species examined in this study, are shown in Fig. 1 and were obtained with the Roth Y02 10-mer primer. The band pattern of each species

Table II. Diosgenin content of leaf samples from 51 *Dioscorea* accessions.

Accession	Total diosgenin content (%)*	Accession	Total diosgenin content (%)*
D. alata E-1-04	0.0874	*D. bulbifera* DB 7087	0.2283
D. alata E-1-06	0.0698	*D. bulbifera* PM3	0.1297
D. alata TDa 289	0.1125	*D. bulbifera* cv. *vera* DB 254	0.2419
D. alata TDa 1349	0.2423	*D. bulbifera* cv. *vera* DB 268	0.0992
D. alata V-1-12	0.0592	*D. caucasica*	0.5233
D. alata V-1-30	0.1220	*D. cayenensis* blackwiss #12	0.7310
D. alata V-1-34	0.0552	*D. cayenensis* yellow AFU yam	0.3074
D. alata V-1-51	0.1210	*D. cayenensis* TDc 95-293	0.4172
D. alata V-1-53	0.0675	*D. cayenensis* TDc 95-294	0.6359
D. alata V-1-55	0.0750	*D. cayenensis* x *rotundata* intermediate	0.1472
D. alata V-1-59	0.0837	*D. composita*	0.3697
D. alata V-1-60	0.0605	*D. deltoidea* cv. "Bombay"	0.0880
D. alata V-1-61	0.0861	*D. deltoidea* cv. "Bayreuth"	0.2052
D. alata V-1-63	0.0581	*D. discolor*	0.0563
D. alata V-1-70	0.0406	*D. japonica*	0.0572
D. alata V-1-87	0.0716	*D. mangenotiana* TDm 2938	0.5366
D. alata V-1-90	0.1079	*D. nipponica*	0.1329
D. alata V-1-95	0.1143	*D. pentaphylla*	0.0716
D. alata V-1-99	0.1268	*D. reticulata*	0.0869
D. batatas	0.0795	*D. rotundata* TDr 608	0.9263
D. bulbifera DB 01	0.1293	*D. rotundata* TDr 747	0.6464
D. bulbifera DB 02	0.1318	*D. rotundata* TDr 3444	0.4477
D. bulbifera DB 06	0.0496	*D. sansibarensis*	0.0541
D. bulbifera DB 10	0.0366	*D.* sp.	0.1002
D. bulbifera DB 18	0.1224	*D. vittata*	0.0444
D. bulbifera DBET 9307	0.1163		

* Sum of diosgenin and yamogenin; values represent the mean of four replicates.

shows distinctive variations in comparison to those of all the other species, with the exception of the lanes representing *D. batatas* and *D. japonica*, respectively, two species, which show noticeable morphological similarities and had previously been assumed to represent two varieties of one species, or, at least, be phylogenetically closely related to each other (Coursey, 1967). In contrast to the latter observation, the DAF patterns of the two accessions *D. deltoidea* cv. "Bayreuth" and *D. deltoidea* cv. "Bombay", respectively, do not show great similarities, which corresponds to their different morphologies. Out of an overall number of 18 *Dioscorea* species investigated, three (*i.e. D. al-*

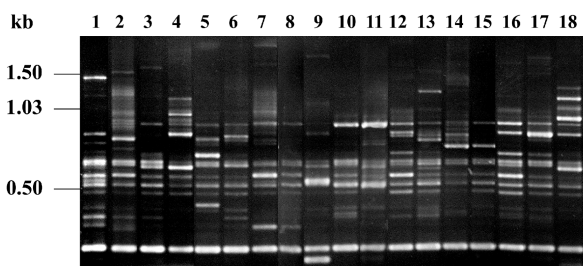

Fig. 1. *Dioscorea* species characterized by DAF. 1, *D. alata*; 2, *D. bulbifera*; 3, *D. caucasica*; 4, *D. cayenensis*; 5, *D. composita*; 6, *D. deltoidea* cv. "Bombay"; 7, *D. deltoidea* cv. "Bayreuth"; 8, *D. reticulata*; 9, *D. discolor*; 10, *D. japonica*; 11, *D. batatas*; 12, *D. mangenotiana*; 13, *D. nipponica*; 14, *D. pentaphylla*; 15, *D.* sp.; 16, *D. rotundata*; 17, *D. sansibarensis*; 18, *D. vittata*.

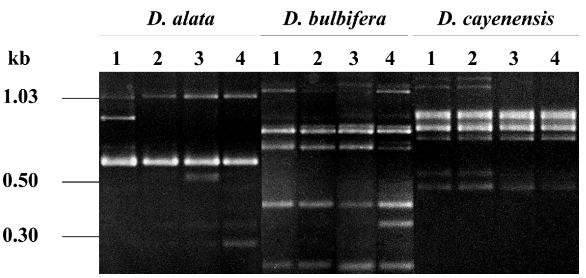

Fig. 2. Intraspecific diversities in selected cultivars of 3 *Dioscorea* species. *D. alata*: V-1-60 (1), V-1-61 (2), V-1-63 (3), V-1-87 (4); *D. bulbifera*: DB 7087 (1), DB 01 (2), DB 02 (3), PM3 (4); *D. cayenensis*: cv. "blackwiss #12" (1), cv. "yellow AFU yam" (2), TDc 95-293 (3), TDc 95-294 (4).

ata, D. bulbifera, and *D. cayenensis*) were selected for monitoring intraspecific genetic variation. The reason for chosing these species was that we considered a number of four cultivars of each species to be the minimum reasonable diversity for intraspecific DAF analysis. The above-mentioned three species were those within our collection to fulfill these requirements.

The results of intraspecific DAF analysis were obtained with the Roth X02 10-mer primer and are shown in Fig. 2. The picture reveals significant differences between the band patterns of each species. A comparison of the intraspecies patterns shows that the strongest bands are present in every lane representing any accession of the respective species, thus offering a rigorous assignment of the tested clones to the corresponding species. Nevertheless, slight genomic differences between the cultivars of each of the three species are obvious, since each lane features weaker bands which appear to be unique in comparison to the other clones.

Discussion

A greenhouse collection of 51 *Dioscorea* accessions originating from multiple locations worldwide, representing 18 species, was screened for the diosgenin content in leaves. Never before a direct comparison of diosgenin contents had been made among a comparable wide collection of *Dioscorea* accessions that were all grown under identical conditions in the same greenhouse. In some publications, *Dioscorea* plants were harvested from different locations and several accessions of the same species contained different amounts of diosgenin (Marker *et al.*, 1943; Wall *et al.*, 1954a, b, 1955, 1957, 1959, 1961). Yet it is not certain, if variations in diosgenin contents correspond to genetic variations of the plants, or if different environmental conditions as well as different stages of the harvested individuals led to the observed divergence. Also, it was not always clearly stated, from which organ the tissue used for analysis was precisely taken. For example, in the series of papers of Wall *et al.* (1952a, b, 1954a, b, 1955, 1957, 1959, 1961) on the screening of over 2000 plants for steroidal and other compounds, it is not clearly defined which parts of *Dioscorea* plants correspond to the listed values. In the first two publications of this series (Wall *et al.*, 1952a, b), detailed descriptions of the applied extraction and analysis procedures are given, and it is mentioned that these techniques were tested on leaf samples of *Agave* plants. However, it remained obscure, which plant organs were actually used throughout the experimentation. In the fourth issue of the same series, *Dioscorea* tubers were tested for alkaloids (Wall *et al.*, 1954b), but again it was never disclosed what tissue actually has been used throughout the whole series of examinations.

Certainly the most astounding confusion in *Dioscorea*-related research arose from quotation of diosgenin concentrations by Coursey (1967), which had been estimated by Marker during his remarkable research on steroid quantification of Mexican yams. Marker *et al.* (1943) published all of the estimated quantitative steroid values on a g per kg basis, whereas Coursey (1967), in his book "Yams", compiled the respective figures in a comparative table together with diosgenin values on a percent basis, quoted from other publications. The consequences of this dramatic error still prevail, since not only 21 out of 53 listed values are tenfold too high, but, most notably, the potential of several *Dioscorea* species as a source for diosgenin was, and still is, terribly overestimated. The persistence of this confusion already led Furmanova and Guzewska (1989) to cite the wrong value of diosgenin content in *Dioscorea bulbifera* from the book "Yams", and this value was consequently adopted by Van Staden and Fowlds (1992).

Another value cited in several publications represents the highest ever estimated diosgenin content in plant material, measured by Ting *et al.* (1980) in *Dioscorea zingiberens*is rhizomes. Extensive investigations on the diosgenin-producing capacity of this species had been carried out by the authors from 1965 to 1980 by screening over 1000 plant accessions, and diosgenin was measured at quantities which never had been found before. However, the method used by Ting *et al.* (1980) raises considerable doubts from today's point of view, since isolated amounts of diosgenin were weighed and compared to the overall dry weight of the respective samples, thus providing a gravimetric content instead of a reliable quantification, which would have been possible by applying chromatographic or spectroscopic methods.

Retrospectively, many published diosgenin values throughout the literature are not reliable at all, and therefore do by no means reflect the synthetic potential of any *Dioscorea* tissue. In view of the worldwide demand of steroids it is mandatory to

define potent sources for steroids. For that reason, we built up a collection of *Dioscorea* species and cultivars and optimized the diosgenin quantification. Also, we paid increased attention to sampling strategies (*e.g.* the plant samples were rigorously taken from the same developmental stage of plants grown under identical ambient conditions), so that comparisons between samples were possible. It was decided to carry out all analyses with leaves instead of rhizome or tuber tissue. The reasons for this choice of material were:

1.) When harvesting leaf tissue, the risk of severe injuries or even irreparable damage is much lower than with major portions of the rhizome or tubers. This was especially important for juvenile plants grown in our greenhouse, since the formation of large tubers can take several years. Since a relatively large amount of material is required for analysis, sufficient quantities of tuber tissue cannot be harvested.

2.) Some of the species maintained in the greenhouse collection remain small even in their adult stages, and therefore do not permit to harvest substantial amounts of rhizome or tuber material.

3.) Leaf material also allows a much better comparability of the results. As has been reported, the content of diosgenin derivatives in tubers and rhizomes of *Dioscorea* increases with the age of the plant individual (Karnick, 1968). A reliable comparison between the diosgenin contents of different individuals could in that case only be achieved if all plant individuals were exactly of the same age.

4.) Finally, the development of *in vitro* cell cultures out of well producing *Dioscorea* cultivars is a major goal of present yam research. *In vitro* cell cultures are more efficiently established from leaf tissue rather than from any subterraneous organ, especially surface-sterilisation is easier to perform, and, last but not least, leaf tissue is the primary location of sterol biosynthesis.

Our results show that the overall diosgenin content in leaves of the tested cultivars ranges from traces to over 0.90% on a dry weight basis. To our knowledge, the total diosgenin content of 0.93%, estimated in leaves of *Dioscorea rotundata* TDr 608, constitutes the highest ever published value of diosgenin content in *Dioscorea* leaves. A comparison with previously published diosgenin concentrations can only be made with reservations, since, for most of the examined species, a quantification of diosgenin in leaf tissue had never been accomplished. Nevertheless, the variation in diosgenin contents within the leaf samples of the whole plant collection falls into a much narrower range than many values retrieved from rhizome or tuber tissues. We assume that diosgenin quantities estimated in leaf tissue represent a more reliable basis for the selection of promising *Dioscorea* clones for *in vitro* propagation.

The results of DNA fingerprinting suggest that DAF is an effective method for the discrimination of different *Dioscorea* species. The comparison of cultivars from one species reveals close genetic similarities. Thus, although slight differences between the band patterns of different accessions exist, the phylogenetic assignment to a certain species can easily be made. For this reason we were able to detect a mislabeling of one accession, which we received as *D. alata* (*D. alata* 7087) and was proved to be a *D. bulbifera* cultivar instead. Consequently, this cultivar was renamed and referred to as *D. bulbifera* DB 7087 for the diosgenin quantification as well as for DAF analysis. The affiliation of the latter clone to the species *D. bulbifera* is clearly depicted in Fig. 2, where the respective lane is the fifth from the left side, located between the patterns of four *D. alata* cultivars at the left, and three *D. bulbifera* cultivars at the right side. Not only does this specific lane not have any band in common with any of the tested *D. alata* cultivars, but every single fragment size is also found at the same length position in at least one of the other three *D. bulbifera* cultivars. The intraspecific DAF analysis also shows the existence of amplification products from more conserved regions, presumably acting as a kind of species-specific fingerprint as they appear in every clone of one species. For *D. alata*, such a higher-conserved replicate of ca. 0.6 kb is present in all four selected clones with nearly identical mass (evidenced by comparable ethidium bromide fluorescence intensity). In *D. bulbifera*, an amplicon of ca. 0.7 kb shows up in each lane, and bands of ca. 0.4 kb length appear in each lane but vary slightly in their respective fluorescence intensities. Within *D. cayenensis*, the two cultivars "blackwiss #12" and "yellow AFU yam" , as well as the two cultivars TDc 95–293 and TDc 95–294 can not be distinguished via DAF. Within these two pairs of accessions, phylogenetic relations seem to be very close, if there is a genetic difference at all. Fingerprinting of the whole *Dioscorea* collection also revealed a misclassification: The fingerprint patterns

of two presumed *D. deltoidea* cultivars suggested, as did their morphology, that the accession received from Bombay, India, and labeled as *D. deltoidea* in fact is a different and unique species, whose identity we could not define. We conclude that DNA fingerprinting contributes to a greater awareness of problems arising from procured *Dioscorea* cultivars for any scientific research.

Our results show that every *Dioscorea* accession in the present study contains diosgenin in the leaves. The estimated diosgenin quantities range below the highest values for subterraneous organs. However, variation among diosgenin contents between different leaf samples is much lower than in tuber tissues, making diosgenin quantification of *Dioscorea* leaf material more comparable, and data for clonal selection more stable. DNA fingerprinting techniques as *e. g.* DAF are mandatory for the proof of cultivar identity.

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