Genetic relationships among accessions of four species of Desmodium and allied genera (Dendrolobium triangulare, Desmodium gangeticum, Desmodium heterocarpon, and Tadehagi triquetrum)

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Research Article

Genetic relationships among accessions of four species of *Desmodium* and allied genera (*Dendrolobium triangulare*, *Desmodium gangeticum*, *Desmodium heterocarpon*, and *Tadehagi triquetrum*)

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Abstract
Random amplified polymorphic DNA markers (RAPD) were used to assess the genetic relatedness among accessions of four species of *Desmodium* and allied genera (*Dendrolobium triangulare*, *Desmodium gangeticum*, *Desmodium heterocarpon* ssp. *heterocarpon*, and *Tadehagi triquetrum*) originating from Northeast Vietnam. Since information on the genetic diversity of these species is deficient, the creation of baseline data is an important means for the development of more sustainable and cost-efficient conservation approaches which eventually result in more comprehensive *ex situ* germplasm collections. The species analyzed are native to tropical and subtropical Asia, Australia, and Oceania and possess a potential as forage and/or medicinal plants. Moderate levels of inter-accession diversity represented by 37.5% and 33.3% of polymorphic fragments (P%) and average Jaccard's similarity coefficients (JSCs) of 0.60 and 0.64 were found in *D. heterocarpon* and *T. triquetrum*, respectively, while moderate to high levels were detected in *D. triangulare* (P% = 52.9 and JSC = 0.61) and *D. gangeticum* (P% = 34.5 and JSC = 0.49). Mantel tests failed to reveal a correlation between geographic and genetic distances. Based on the results of this study, baseline data for further marker-assisted research are generated and future collecting and *ex situ* conservation strategies for the species studied are discussed.

Keywords: cover crop, *Desmodium* spp., *ex situ* conservation, forage, genetic relatedness, medicinal plants, Northeast Vietnam, RAPD

Zusammenfassung
Random Amplified Polymorphic DNA Markers (RAPD) wurden verwendet, um die genetische Verwandtschaft zwischen den Herkünften von vier aus Nordostvietnam stammenden Arten der Gattung *Desmodium* und verwandter Gattungen (*Dendrolobium triangulare*, *Desmodium gangeticum*, *Desmodium heterocarpon* ssp. *heterocarpon* und *Tadehagi triquetrum*) zu untersuchen. Da Informationen über die genetische Diversität dieser Arten nur unzureichend vorhanden sind, ist die Schaffung von Grundlagendaten eine wichtige Voraussetzung für die Entwicklung nachhaltigerer und kosteneffizienterer Konservierungsansätze, die schließlich zu umfassenderen *ex situ* Sammlungen führen sollen. Die analysierten Pflanzenarten sind im tropischen und subtropischen Asien, Australien und Ozeanien heimisch und besitzen ein Potential als Futter- und/oder Medizinalpflanzen. Ein mittlerer Diversitätsgrad zwischen den Herkünften, dargestellt durch 37,5% und 33,3% polymorpher Fragmente (P%), und durchschnittliche Jaccard Koeffizienten (JSC) von 0,60 und 0,64, fanden sich bei *D. heterocarpon* bzw. *T. triquetrum* während ein mittlerer bis hoher Grad der Diversität bei *D. triangulare* (P% = 52,9 und JSC = 0,61) und *D. gangeticum* (P% = 34,5 und JSC = 0,49) festgestellt wurde. Mantel Tests konnten keine Korrelation zwischen geographischer und genetischer Distanz nachweisen. Basierend auf den Ergebnissen dieser Studie werden Grundlagendaten für die weitere Forschung im Bereich molekularer Marker bereitgestellt und zukünftige Sammelstrategien und *ex situ* Konservierungsmaßnahmen der untersuchten Arten diskutiert.

Schlüsselwörter: Bodendecker, *Desmodium* spp., *ex situ* Konservierung, Futterpflanzen, genetische Verwandtschaft, Medizinalpflanzen, Nordostvietnam, RAPD

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Introduction

Within the Leguminosae, the tribe Desmodieae (Benth.) Hutch. is recognized for its substantial contribution to forage production in the tropics [43]. It comprises several species of agronomic interest such as *Dendrolobium triangulare* (Retz.) Schindl. (syn. *Desmodium triangulare* [Retz.] Merr.), *Desmodium gangeticum* (L.) DC., *Desmodium heterocarpon* (L.) DC. ssp. *heterocarpon*, and *Tadehagi triquetrum* (L.) H. Ohashi (syn. *Desmodium triquetrum* (L.) DC.) (Fig. 1). Due to their ability to adapt to low-fertility soils and to tolerate water drought, these species represent valuable plant genetic resources for forage production, soil protection and improvement in marginal smallholder farming systems of subhumid and humid tropical regions [10, 20, 41]. Moreover, the four legume species included in this study have a long history in traditional Asian health care [30, 33, 46].

With the exception of *D. triangulare,* a woody, perennial shrub, all analyzed species are woody herbs or subshrubs that mainly occur in fallow areas, thickets, forest borders, or along roadsides [4, 29]. The genus *Desmodium* and its allied genera, members of the subtribe Desmodiinae, are mainly native to the tropics and subtropics. Ohashi [28] records two centers of geographic distribution and differentiation: Southeast Asia and Mexico. According to Williams [47] Southeast Asia is a center of legume diversity. There, systematic exploration of native legume genetic resources started only about two decades ago when a series of collecting missions under the auspices of CIAT (International Center for Tropical Agriculture) was carried out in Malaysia, Indonesia, tropical China, Thailand, Papua New Guinea, and Vietnam. However, the mountainous and highly diverse north of Vietnam was not visited during previous collecting missions [37, 38]. At the same time, plant genetic resources of the North Vietnamese mountains are increasingly threatened due to ecosystem transformation and growing demand of natural resources in the wake of high population pressure [45]. For these reasons the area was chosen as the collection target in this study.

Molecular marker techniques have become increasingly important in conservation biology. Information about the underlying genetic diversity of species is essential for sustainable use and efficient conservation efforts of plant genetic resources [21, 40]. Unfortunately, such information is still inadequate concerning species of *Desmodium* and allied genera, especially at the DNA level. The traditional methodological approach to analyzing genetic diversity is based on morphological and/or agronomic traits. However, results of such studies might be biased by confounding environmental factors that influence the phenotype. Apart from *D. heterocarpon*, all *Desmodium* species so far analyzed were merely subjected to taxonomic, agronomic or, in the case of *D. gangeticum,* chemical studies [9, 22, 28, 41]. Isozyme electrophoresis allowed

distinguishing *D. heterocarpon* ssp. *ovalifolium* from *D. heterocarpon* ssp. *heterocarpon* [27] but failed to discriminate the former at genotype level [17]. In a recent study, genetic diversity of a germplasm collection of *D. heterocarpon* ssp. *ovalifolium* held by CIAT was analyzed and a tentative core collection built up on the basis of random amplified polymorphic DNA (RAPD) marker data [6].

RAPDs provide a simple, fast, and cost efficient methodology to primarily screen the level of genetic variation of a great number of yet unknown genotypes [48]. Even though RAPDs are frequently criticized for unreliable reproducibility [15], dominance [25], and comigration [35], they have proven to be a useful tool in studies analyzing genetic variation in a substantial number of tropical legume species (e.g., [8], [16], [23]).

For these reasons, RAPD markers were used in the present study to determine the genetic relatedness among accessions of four *Desmodium* and allied genera collected in Northeast Vietnam. The RAPD methodology applied will also be useful for screening duplication and establishing core collections in gene banks.

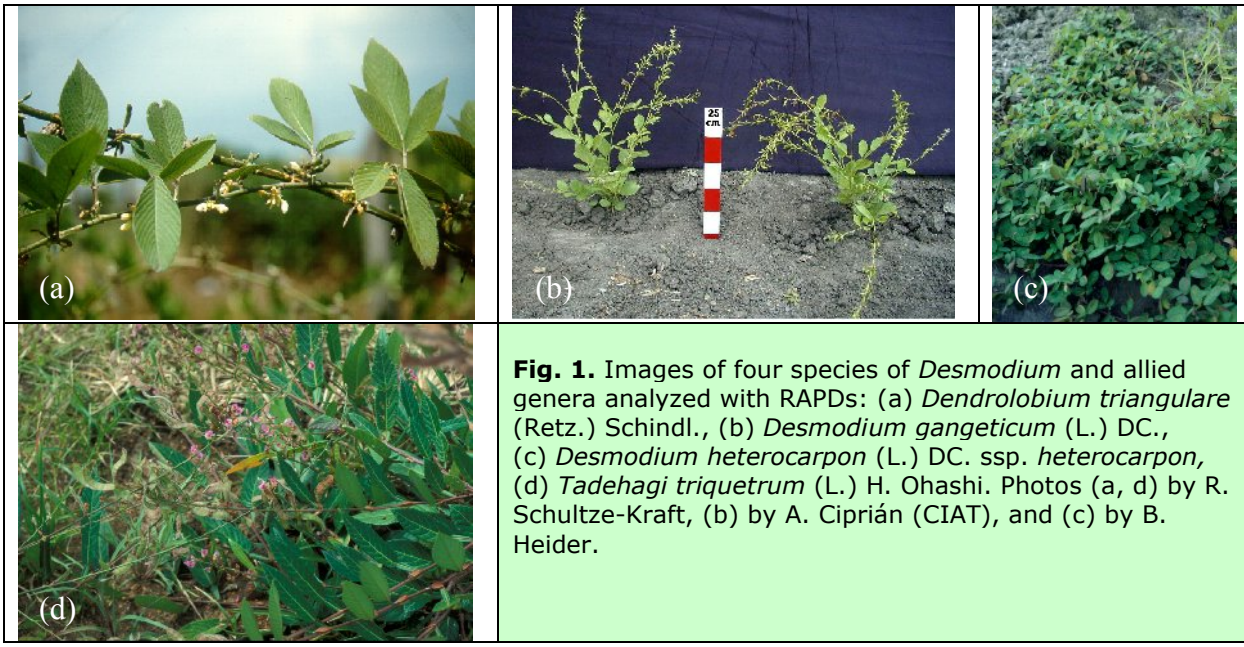

Fig. 1. Images of four species of *Desmodium* and allied genera analyzed with RAPDs: (a) *Dendrolobium triangulare* (Retz.) Schindl., (b) *Desmodium gangeticum* (L.) DC., (c) *Desmodium heterocarpon* (L.) DC. ssp. *heterocarpon,* (d) *Tadehagi triquetrum* (L.) H. Ohashi. Photos (a, d) by R. Schultze-Kraft, (b) by A. Ciprián (CIAT), and (c) by B. Heider.

Methods

Plant material - Germplasm of the four species was collected in 1999 and 2000 from native populations within their natural habitats in Bac Kan province, Northeast Vietnam (Fig. 2). At each collecting site ecogeographic and plant specific passport data were recorded including geographic position, elevation, habitat, topography, soil, and site characteristics as well as plant-related information (e.g., plant vigor, occurrence of pests and diseases). A total of 35 *Dendrolobium triangulare*, 37 *Desmodium gangeticum*, 66 *Desmodium heterocarpon*, and 24 *Tadehagi triquetrum* accessions were included in this study. All accessions are combined (bulked) seed samples in order to represent the natural population at the sites where they were collected.

Ten plants of each accession were propagated from scarified seeds and maintained under greenhouse conditions. Fresh leaf material was sampled from six-month-old plantlets. In the case of *T. triquetrum* and *D. heterocarpon*, genetic analyses were performed on a maximum number of 10 individual plants per accession in order to obtain sufficient leaf material for DNA extraction. Growth of *D. gangeticum* and *D. triangulare* was more vigorous, yet few individuals failed to survive. In order to standardize methodology, the number of individuals per bulk was limited to five plants per accession forming the *a* bulk. The surplus was placed in the *b* bulk. DNA of *a* and *b* bulks was separately isolated and analyzed. The division into an *a* and *b* bulk was also used to approximate the ideal number of plants per bulk.

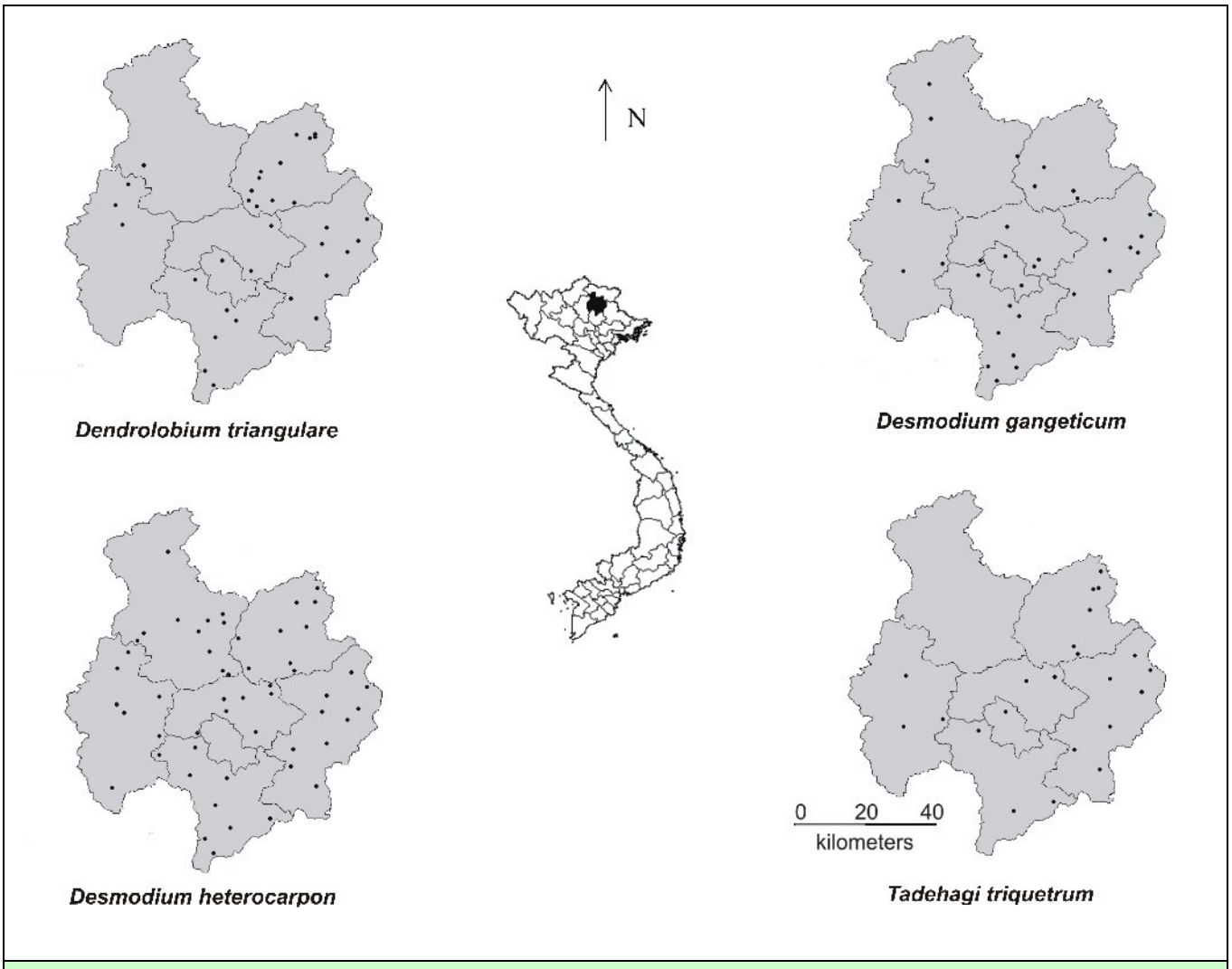

Fig. 2. Collecting sites of *Desmodium* and allied genera in Bac Kan province, Northeast Vietnam.

DNA isolation and amplification - Genomic DNA was extracted from 100 mg of fresh leaf tissue. Samples were frozen in liquid nitrogen and subsequently homogenized. Three out of the four species (*D. gangeticum, D. heterocarpon,* and *T. triquetrum)* were analyzed using a small-scale DNA isolation method (Plant DNA Mini Kit EZNA from Peqlab). In the case of *D. triangulare*, the established method had to be modified since several alternative extraction protocols were tested but failed to extract good quality DNA. Therefore DNA extraction and purification were finally performed with the DNeasy Plant Mini Kit from Qiagen which provided the best DNA extraction results. DNA concentrations were measured (Gene Ray photometer; Biometra) and standardized against known concentrations of λ-DNA (Roche Diagnostics) on agarose gels. After quantification DNA was diluted in TE buffer to a final concentration of 5 µg DNA μl^{-1}.

Polymerase chain reaction (PCR) amplifications were performed in reaction mixtures containing 10 ng template DNA, 0.2 µM primer, 100 µM dNTP´s, 10 mM PCR buffer, 1.5 mM $MgCl_2$, and 1 unit *Taq*-polymerase in a total volume of 10 µl. Amplifications were carried out in a thermo-cycler (T Gradient, Biometra) with the following program: initial denaturation step of 3 min at 94 °C followed by 34 cycles of 1 min at 94 °C, 1 min at 36 °C, 2 min at 72 °C, and a final extension step of 5 min at 72 °C. DNA fragments were resolved by electrophoresis in 1.5% agarose gels run at 4 V cm^{-1}. Gels were stained with ethidium bromide, visualized under UV-light, and photographed by a CCD video camera (Raytest). To ensure reproducibility of banding patterns, each amplification reaction was repeated twice. Four primer kits (L, M, N, and U; Roth), each comprising 20 decamer primers, were screened for each of the four analyzed species. Only those primers were selected that showed well-defined and reproducible banding patterns (Table 1).

Data analysis - Presence or absence of amplified products was scored as discrete characters (1 = presence and 0 = absence) across all accessions and for each primer. The resulting binary matrices were used to calculate Jaccard's similarity coefficients (JSCs) with the aid of SIMQUAL (Similarity for Qualitative Data) routine. Based on JSCs, dendrograms were constructed by means of the unweighted pair group method with arithmetic averages (UPGMA) employing SAHN (Sequential, Agglomerative, Hierarchical, and Nested clustering). These analyses were performed using the software package NTSYS-pc, version 1.80 [36]. Based on the calculation of pairwise binary genetic distances for dominant data [5, 14] a Mantel test (999 permutations) was applied to analyze correlations between genetic and geographical distances for each accession using GenAlEx V5 [31].

Results

Dendrolobium triangulare (Retz.) Schindl.

RAPD polymorphism - In the analysis of 35 *D. triangulare* accessions, four primers were used that generated a total of 17 RAPD fragments of which 52.9% were polymorphic. Each primer amplified four (L-16, M-10, and N-10) to five (L-15) markers, an average of 4.25 markers per primer and 2.25 polymorphic markers per primer, which ranged in size from 350 to 1300 basepairs (bp; Table 1). Examples of RAPD profiles due to primer L-15 are shown in Fig. 3.

Table 1: Oligonucleotide primers employed in RAPD analysis, their sequence, number of bands obtained per primer, size range of bands, and specific size of polymorphic (polym.) fragments.

Primer code	Sequence (5' to 3')	Number of bands		Size (bp)	Specific size (bp) of polym. fragments
		Polym.	Monom.	min-max	
Dendrolobium triangulare					
L-15	AAGAGAGGGG	4	1	500-1000	700, 800, 900, 1000
L-16	AGGTTGCAGG	1	3	490-1000	490
M-10	TCTGGCGCAC	1	3	450-1300	1300
N-10	ACAACTGGGG	3	1	350- 700	500, 600, 700
Desmodium gangeticum					
L-01	GGCATGACCT	2	2	450- 900	520, 750
L-15	AAGAGAGGGG	2	3	500-1300	620, 750
L-18	ACCACCCACC	2	2	250-1200	700, 1200
M-06	CTGGGCAACT	1	5	200-1500	480
M-10	TCTGGCGCAC	1	2	750-1400	1100
M-12	GGGACGTTGG	2	5	320-1300	600, 650
Desmodium heterocarpon					
L-11	ACGATGAGCC	1	5	290-1300	900
L-15	AAGAGAGGGG	3	2	400-1100	650, 750, 1100
L-19	GAGTGGTGAC	2	1	400- 900	750, 900
N-01	CTCACGTTGG	2	4	400-1000	700, 900
N-09	TGCCGGCTTG	1	3	500-1000	900
Tadehagi triquetrum					
L-13	ACCGCCTGCT	1	4	350-1200	450
M-12	GGGACGTTGG	2	3	450-1200	900, 1100
M-18	CACCATCCGT	2	3	350-1250	1200, 1250
N-10	ACAACTGGGG	3	1	350- 650	520, 650, 700
U-09	CCACATCGGT	1	3	400-1000	1000
U-20	ACAGCCCCCA	1	6	350-1300	700

JSCs based on RAPD variation - A similarity matrix based on JSCs was constructed to estimate the relatedness among accessions. Generally, JSCs of all analyzed species ranged from 0 to 1. In the case of *D. triangulare*, the lowest value (JSC = 0) was found in comparisons of accession 493*a* to accession 744*a* and 873*b*, respectively. Mean JSC was 0.61 indicating that a medium to high level of genetic similarity was identified among *D. triangulare* accessions. Out of a total of 35 accessions, 23 were divided into *a* and *b* bulks yielding 12 accessions, in which *a* and *b* bulks proved to be identical. However, 65.7% were divergent.

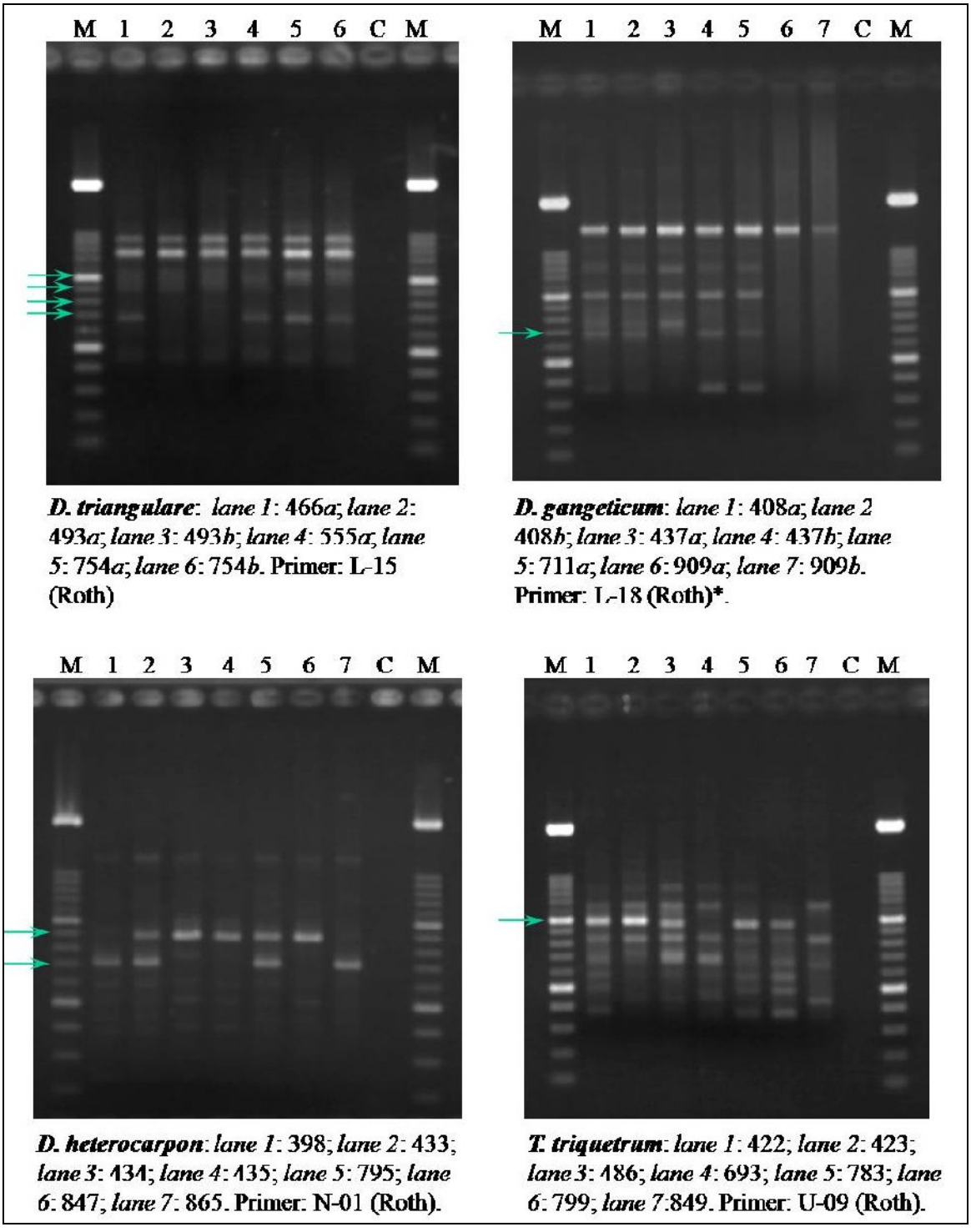

Fig. 3. Examples of RAPD profiles among accessions of *Dendrolobium triangulare*, *Desmodium gangeticum*, *D. heterocarpon*, and *Tadehagi triquetrum* obtained with the respective primers. Numbered lanes display the RAPD profile of different accessions; lane M: molecular weight marker (100 bp DNA-ladder, Roche Diagnostics); lane C: negative control ($H_2O_{dest.}$ instead of template DNA). * Polymorphism at 1200 bp not shown on this gel.

UPGMA cluster analysis - The dendrogram (Fig. 4) depicts a diverse and complex clustering. Accessions of *D. triangulare* were classified into seven main clusters (I to VII). *A* and *b* bulks of the same accessions were frequently located in different subclusters with very few exceptions positioned within the same main cluster. The first and second cluster comprised 73% of all accessions. No tendency to group in accordance to collecting regions was detected. A **Mantel test** resulted in a coefficient of correlation between genetic and geographic distances of R = 0.095 (P = 0.042).

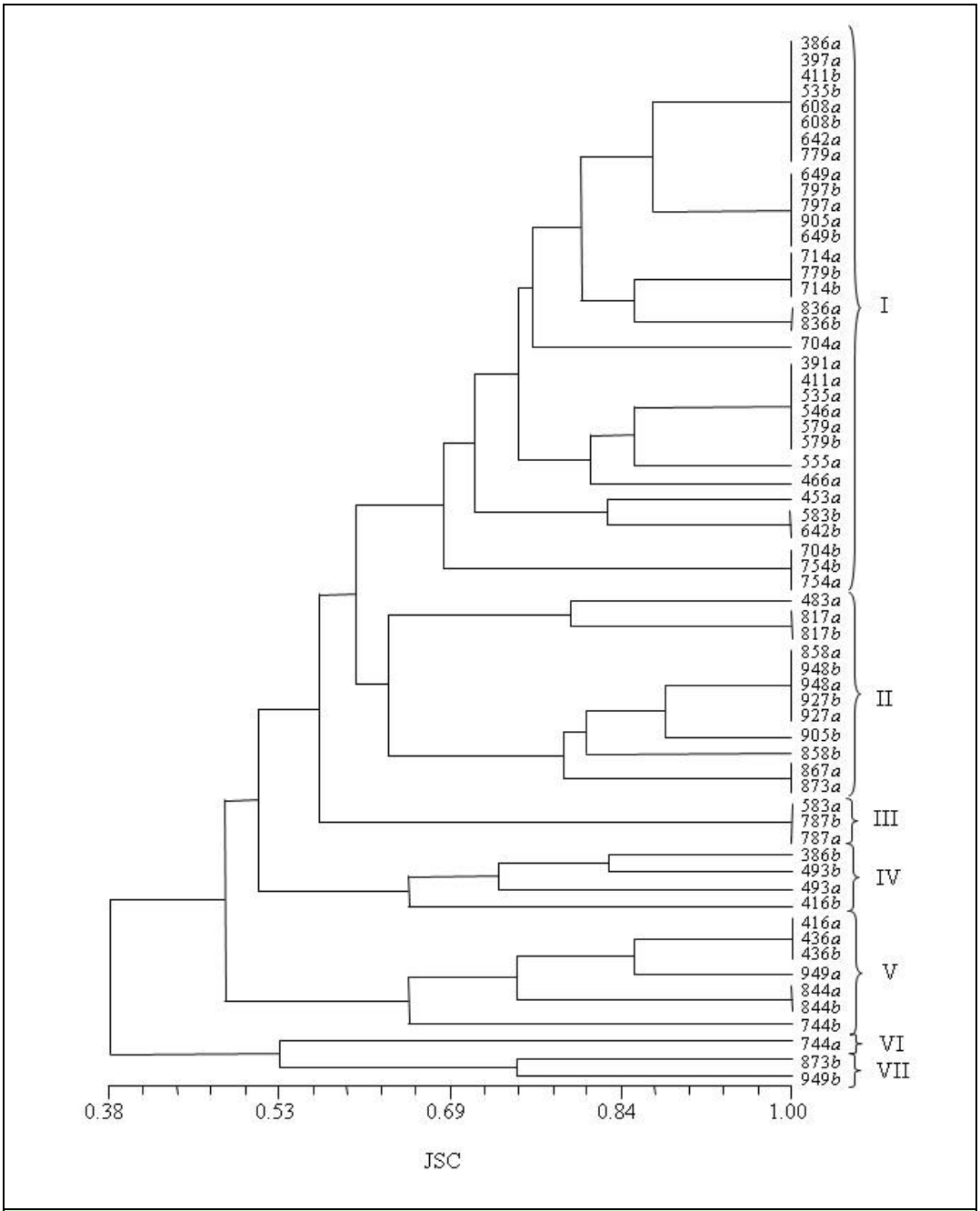

Fig. 4. UPGMA dendrogram illustrating genetic relationships among 35 accessions of *Dendrolobium triangulare.* The numerical scale of JSCs indicates increasing similarity.

Desmodium gangeticum (L.) DC.

RAPD polymorphism - A total of six primers were used to screen 37 accessions of *D. gangeticum*. These selected primers amplified 29 bands, of which ten were polymorphic (34.5%). The number of amplified fragments varied from three (M-10) to seven (M-12) with an average of 4.83, and fragment size from 200 to 1500 bp (Table 1). The typical RAPD banding pattern obtained with primer L-18 is presented in Fig. 3.

JSCs based on RAPD variation - RAPD-based JSCs ranged from 0 to 1. Average JSC was 0.49, the lowest value among all species analyzed in this study. Out of 37 accessions, 34 were split into *a* and *b* bulks. Fewer than half (43.2%) of all *b* bulk results confirmed the respective *a* bulk data. A moderate to high level of genetic diversity was found among the analyzed *D. gangeticum* accessions.

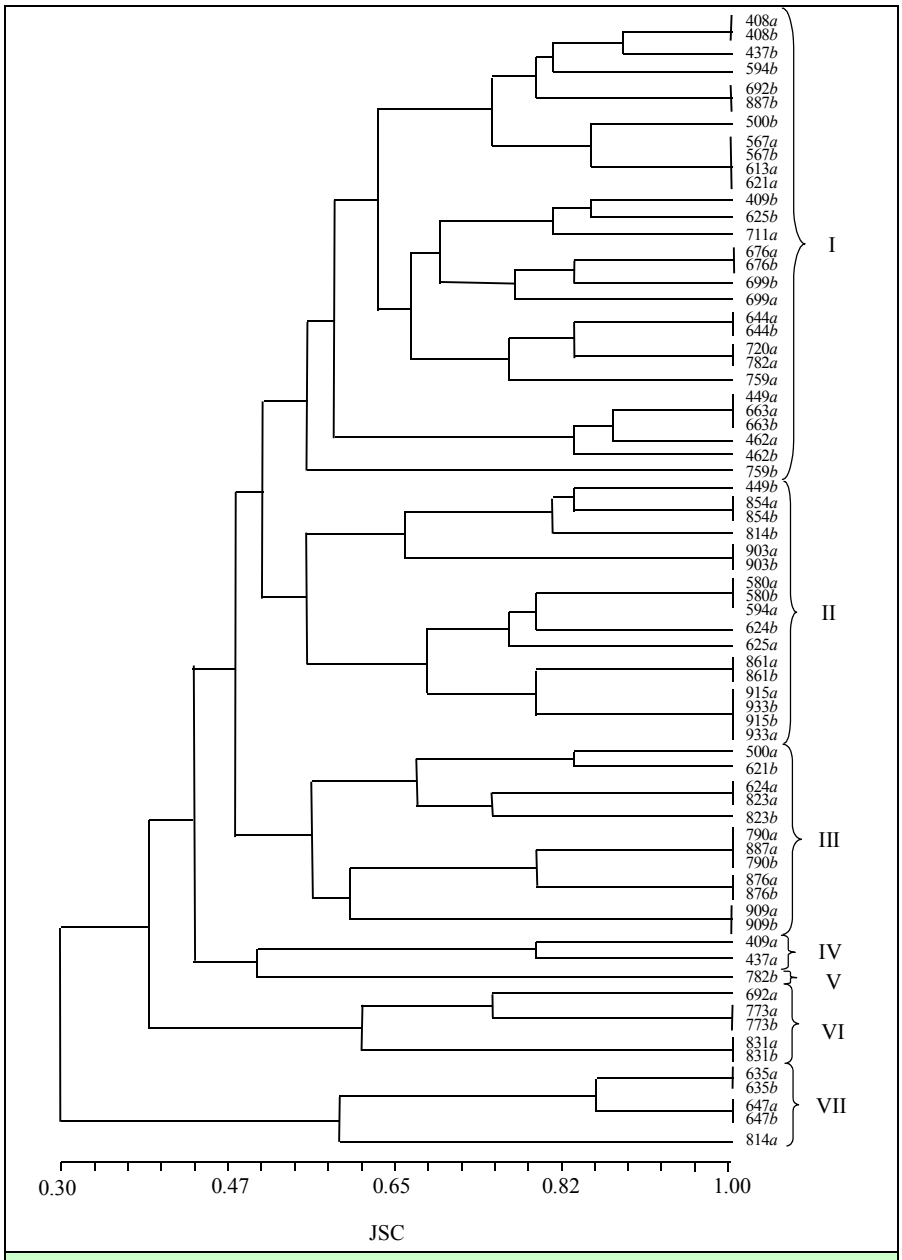

Fig. 5. UPGMA dendrogram illustrating genetic relationships among 37 accessions of *Desmodium gangeticum.* The numerical scale of JSCs indicates increasing similarity.

UPGMA cluster analysis - A complex dendrogram visualizes how the *D. gangeticum* accessions are grouped on the basis of JSCs (Fig. 5). Seven main clusters shared more than 30% of all alleles. The clustering does not allow concluding whether there is a correspondence to geographical boundaries or origin. A **Mantel test** showed no correlation between genetic and geographic distances (R = 0.032; P = 0.186).

Desmodium heterocarpon (L.) DC. ssp. *heterocarpon*

RAPD polymorphism - The five primers selected to detect polymorphism among 66 *D. heterocarpon* accessions yielded a total of 24 RAPD markers of which nine (37.5%) were polymorphic. The total number of scored products per primer ranged from three (L-19) to six (L-11) with an average of 4.8. Molecular mass of bands varied from 290 to 1300 bp in size (Table 1). Examples of the polymorphism detected with primer N-01 are shown in Fig. 3.

JSCs based on RAPD variation - Among the investigated *D. heterocarpon* accessions an intermediate level of genetic similarity was found. RAPD based JSCs varied from 0 to 1 with a mean of 0.60.

UPGMA cluster analysis - Two main clusters (I and II) can be distinguished that deviate at a JSC of 0.27 (Fig. 6). Accessions within each of the two clusters share between 61% (lower division) and 67% (upper division) of all alleles. Genetic differentiation among accessions of the two genetically distinct main clusters (I and II) is substantial though it is not related to geographic distances (**Mantel test**: R = 0.05; P = 0.187).

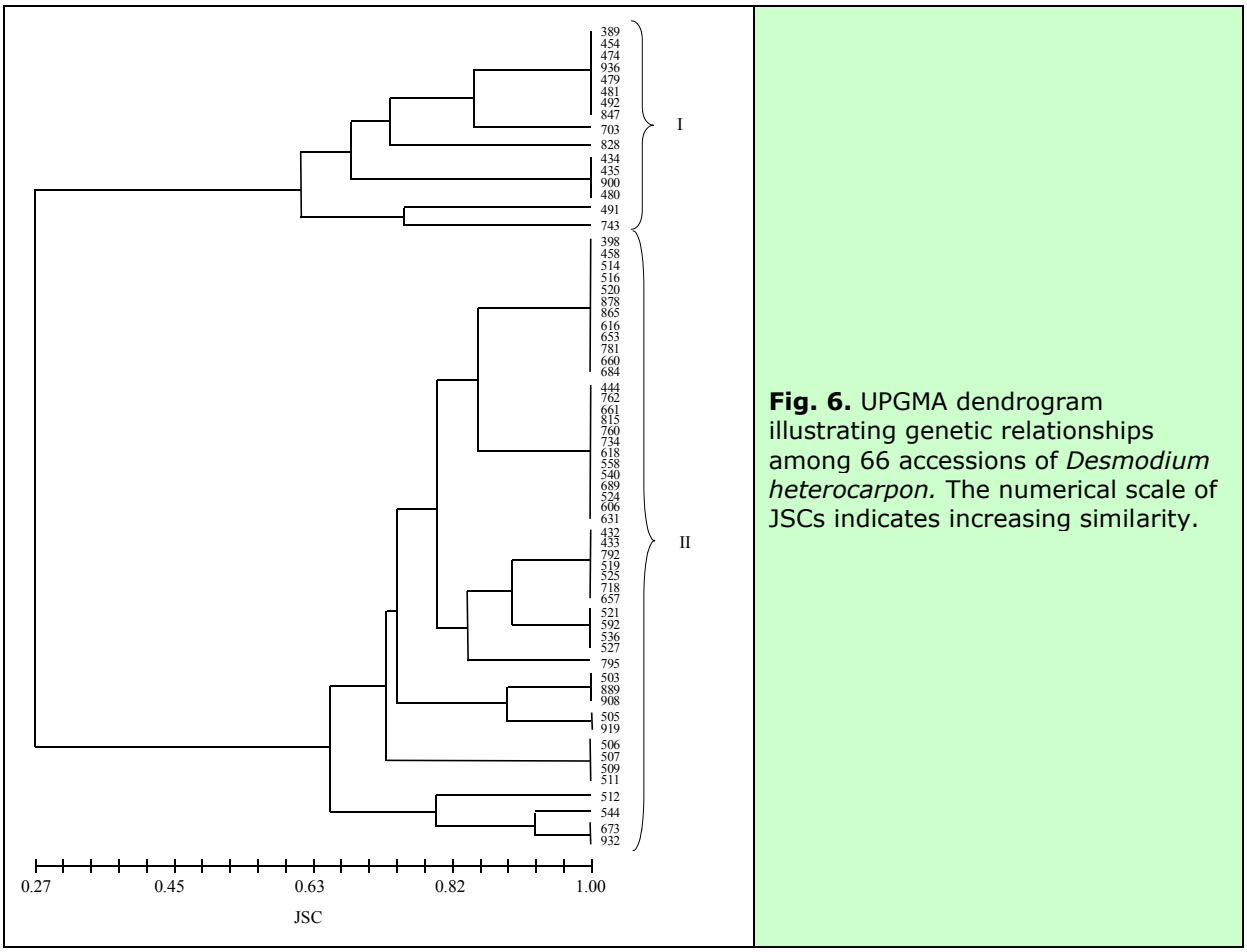

Fig. 6. UPGMA dendrogram illustrating genetic relationships among 66 accessions of *Desmodium heterocarpon.* The numerical scale of JSCs indicates increasing similarity.

Tadehagi triquetrum (L.) H. Ohashi

RAPD polymorphism - The 24 *T. triquetrum* accessions were analyzed using six primers that produced 30 RAPD markers ranging in size between 350 and 1300 bp. The total number of amplified bands per primer varied between four (N-10 and U-09) and seven (U-20) with an average of five bands per primer (Table 1). Mean percentage of polymorphic bands of all primers was 33.3%. Of the polymorphic fragments, three were unique and occurred only in accession 918. An example of the amplification products obtained with primer U-09 is shown in Fig. 3.

JSCs based on RAPD variation - Jaccard's similarity coefficients ranged from 0 to 1 with a mean of 0.64. The lowest similarity value was exclusively observed in comparisons of accession 918 with other accessions.

UPGMA cluster analysis - The UPGMA dendrogram divided into four main clusters (I-IV, Fig. 7). The first branch separated the outlier accession 918 from the remaining samples at a JSC of 0.04. Accession 918 formed an isolated cluster (IV), most distinct from all other accessions. The division of the main branch detached into an upper (I and II) and a lower cluster (III) at a JSC of 0.45. The UPGMA dendrogram visualises an intermediate level of similarity among *T. triquetrum* accessions. Clustering, however, did not coincide with geographic distances. A **Mantel test** rendered a coefficient of correlation of R = 0.135 (P = 0.107).

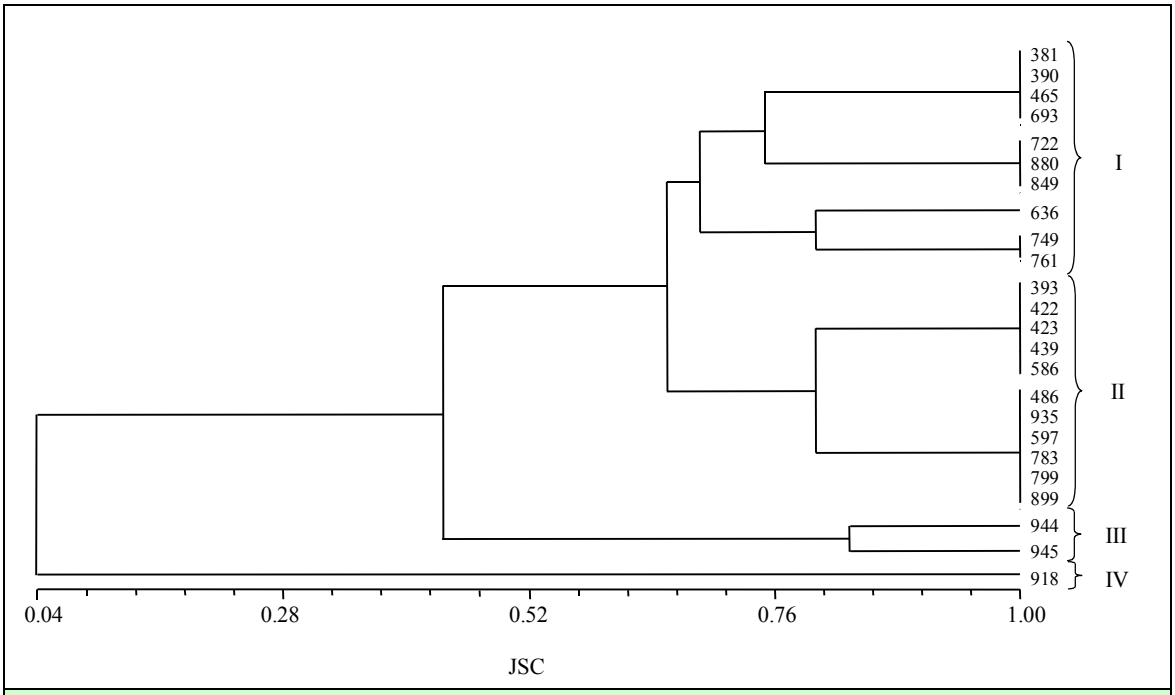

Fig. 7. UPGMA dendrogram illustrating genetic relationships among 24 accessions of *Tadehagi triquetrum.* The numerical scale of JSCs indicates increasing similarity.

Discussion

RAPD analyses were useful to determine genetic relatedness among accessions of four species of *Desmodium* and allied genera collected in North Vietnam. Moderate levels of diversity existed among accessions of *D. heterocarpon* and *T. triquetrum* while moderate to high levels were found in *D. gangeticum* and *D. triangulare*.

The level of polymorphism observed among accessions of *D. gangeticum* (34.5%), *D. heterocarpon* (37.5%), and *T. triquetrum* (33.3%) was among the lowest compared to many studies of tropical herbaceous legume species. Liu [23, 24] reported 68.9% of polymorphism among *Lablab purpureus* and 86.6% among *Stylosanthes scabra* genotypes. RAPD analyses of *Vigna angularis* (49%), *Pueraria montana* var. *lobata* (54.3%), and *P. phaseoloides* (45.5%) yielded slightly higher levels of polymorphism [13, 49]. Comparisons of *D. triangulare* (52.9%) with RAPD marker studies of other tropical legume trees and shrubs resulted in a relatively high level of polymorphic loci for this species. While higher levels of polymorphism were reported for *Cratylia argentea* (91.8%) [1], and *Flemingia macrophylla* (95%) [12], lower levels were found in Argentinian *Acacia* species (19.1%) [3] and *Prosopis* species (28.3%) [16]. However, it should be recognised that the chosen primer selection approach may not have resulted in unbiased estimates of levels of polymorphism. Preliminary primer screens were run on a selection of 10 accessions per species and only those primers that generated polymorphic fragments were selected. In the case of uniform amplification, primers were discarded and not used for full screening of all accessions which might have interfered with data accuracy.

Average JSCs obtained in this study (*D. heterocarpon*, 0.60, *T. triquetrum*, 0.64, and *D. gangeticum*, 0.49) were comparable to previous RAPD marker studies of herbaceous legumes. Xu et al. [49] found JSCs of 0.73 in cultivated forms and 0.60 in wild and weedy populations of *V. angularis*, while Yee et al. [50] and Heider et al. [13] recorded JSCs of 0.52 in *V. angularis* and *P. phaseoloides,* respectively. Results of a recent molecular marker study showed a comparatively low JSC in *Pueraria montana* var. *lobata* (0.35) [13]. RAPD analyses of tropical tree and shrub legumes revealed average JSCs that were slightly higher in *F. macrophylla* (0.67) [12] than in *D. triangulare* (0.61). However, the fact that a large number of previous studies on genetic diversity of tropical legumes did not disclose the overall JSC, used different diversity indices, or employed different molecular marker techniques, represents an obstacle to direct comparisons.

Several authors experimented with the optimal number of individual plants that were pooled in order to reduce the sensitivity of RAPDs to DNA sequence variation and thus to ensure a representative estimation of genetic diversity in heterogeneous populations [7, 18, 19]. The recommended number of individuals per bulk ranged between three and 20. Based on these references, bulk sizes in the study of *D. heterocarpon* and *T. triquetrum* was adjusted to 10 individuals in each bulk. Due to low germination rates and low seedling vigor, the number of surviving greenhouse individuals restricted the bulk sizes of *D. triangulare* and *D. gangeticum* to five individual plants each (*a* bulk). Any additional plants formed *b* bulks that consisted of up to five individuals. The fact that 70.4% of *a* and *b* bulks of *D. triangulare* and 43.2% of *D. gangeticum* were not identical and therefore did not coincide in the same cluster, illustrates clearly that bulk sizes were too small to dilute rare alleles. In general, Desmodieae are assumed to be predominantly self-pollinated, but outcrossing rates, ranging from 0.01% to 10%, are also likely to occur as in other tropical legumes [26, 32]. Given the rudimentary knowledge available on breeding systems of the analyzed species, the degree of heterozygosity inherent in accessions of *D. triangulare* and *D. gangeticum* seems to be much higher than originally assumed. Seeds were sampled as accessions representing the natural population from which they were collected. The aim of collecting accessions is to capture the entire range of genetic variation, as much as possible, in a given population [2]. Thus individual seedlings were bulked for RAPD analysis to represent the genetic constitution of the

original accession. The exclusive use of bulk samples, however, does not allow the use of statistical parameters of population genetics and impedes the assessment of the species' breeding system. There was no substantial information available on the reproduction mode of the four studied species. However, such information is decisive for the amount and partitioning of genetic variation in and among populations [11]. Therefore, in order to draw firm conclusions on intra-accession diversity, individual plants composing an accession should additionally be analyzed in future studies, a measure that might aid in improving data quality and thereby contribute to optimizing collection and conservation management.

Implications for conservation

Desmodium and allied genera play an important role in different research and development endeavors to address declining soil fertility, erosion, overgrazing, and dry-season forage constraints in marginal areas of the tropics and subtropics. *Desmodium heterocarpon* ssp. *heterocarpon* attracted research interest due to its ability to persist in highly productive grass/legume mixtures in humid subtropical and tropical regions, while tolerating heavy grazing, acid soils, drought, temporary water-logging, and moderate frost periods [10]. The carpon desmodium cultivar "Florida" of this species obtained economic significance as a forage legume in peninsular Florida [39]. *Tadehagi triquetrum* (L.) H. Ohashi is agronomically interesting because of its capacity to provide crude protein for livestock feeding during dry seasons and for its good adaptation to acid, low-fertility soils [42]. *Dendrolobium triangulare* (Retz.) Schindl. provides fuelwood and is suitable for soil protection and improvement. It has minor forage potential due to variable levels of condensed tannins [22]. *Desmodium gangeticum* (L.) DC. was found to be highly productive in shady environments such as under plantation crops [41]. Particularly, *D. gangeticum* is considered a plant resource of pharmaceutical importance which serves as a remedy for diarrhea, dysentery, and several eye diseases. These curative properties are due to a number of alkaloids, a pterocarpanoid (gangetin), flavons, and isoflavanoid glycosides as active compounds with antifungal, antileishmanial, antioxidant, antiradical, and anticholinesterase activities [e.g., 9].

The mountainous north of Vietnam experienced dramatic environmental and social changes during the last decades threatening the biological diversity of the region [45]. Desmodieae as well as other wild-growing plant species are subjected to genetic erosion because of habitat destruction and resource overexploitation as a consequence of increasing demographic pressure. Since the vast majority of tropical forage legume cultivars are unimproved selections from wild populations [34], the enormous yet untapped legume diversity of the North Vietnamese mountainous regions should be explored in order to avoid reliance on a narrow germplasm base. This includes further germplasm collecting as well as efficient *ex situ* conservation measures. Conservation of plant genetic diversity in gene banks requires balancing carefully between numerically extensive collections and the feasibility of maintaining and managing germplasm storage. Knowledge about genetic diversity in a target taxon is also indispensable when considering the establishment of a core collection or eliminating redundancy in collections [21, 40].

The heterogeneous clustering of *a* and *b* bulks in *D. triangulare* (Fig. 4) and *D. gangeticum* accessions (Fig. 5) imposes difficulties to the establishment of an *ex situ* conservation approach for these species. But generally, genotypes that are redundant may be removed from *ex situ* storage while outliers and particularly distinct accessions should be included. Clusters in which numerous extremely related accessions occur may be condensed to subsamples. However, such selection must be carried out with circumspection as RAPD diversity does not necessarily reflect diversity at quantitative trait loci, and important genotype characteristics might become lost [44]. Thus morphological plant characteristics should also be considered before discarding

germplasm. In the case of *D. triangulare* and *D. gangeticum*, each cluster VII certainly encompasses the most distinct accessions (Figs. 4 and 5). Accessions that did not represent unique genotypes, i.e., those that formed clusters at a JSC of 1, are candidates for removal. The *D. triangulare* dendrogram contains several of these, particularly in clusters I and II. The first three clusters of *D. gangeticum* could also be reduced.

Within the *D. heterocarpon* (Fig. 6) collection accessions 491, 512, 544, and 743 were the most distinct accessions and therefore suggest themselves for *ex situ* conservation. Apart from these, a representative subsample of each cluster, eliminating the numerous duplicates, would suffice.

A comparison of passport data between the two main clusters of *D. heterocarpon* revealed that accessions forming the upper division were collected from humid and frequently shady habitats growing in heavy soils characterized by increased nitrogen, calcium, magnesium, and organic matter contents while accessions from the lower division grew in poorer soils and in more marginal, sun-prone, and therefore drier environments (data not shown). Thus it appears that the two main divisions consist of two different ecotypes.

High genetic differentiation was found among three of 24 *T. triquetrum* accessions (944, 945, and 918), while the remaining accessions harbored high similarity values among them. The outlier (918) of *T. triquetrum* exhibited three unique markers and as a consequence may contain unique genetic characteristics, which indicates that significant distinctiveness was captured here. However, neither taxonomic plant characteristics nor collecting site-related passport data could explain this distinctiveness. In general, data show that *T. triquetrum* represented the least diverse collection among the four species analyzed but also the smallest. Therefore, it seems advisable to conduct further, more extensive collecting missions at larger distances in order to collect more genetic diversity that would qualify for *ex situ* conservation. As for the samples collected in Bac Kan province, the outlier, accessions comprising cluster III, and a representative subsample of clusters I and II (Fig. 7) should be included if aiming at efficient *ex situ* conservation.

Future collecting missions should target populations at large distances covering altitudinal gradients in order to ensure that the ecological and geographic ranges of the respective species are included [2]. Outcrossing must be assumed in all four species studied. Hence, individuals within distant populations should be comprehensively collected instead of extracting a few individuals from many populations at short distances. A manageable and efficient *ex situ* conservation approach aims at capturing maximum genetic diversity within stored material, comprising outliers and genetically distinct germplasm, and representative subsamples of very similar or even redundant genotypes. Ideally such *ex situ* conservation would be based on both molecular and morphological data in order not to lose valuable adaptive variation.

Concluding remarks

Generated data showed that RAPD markers, despite their widely discussed disadvantages, are suitable tools to scan anonymous DNA of species with high secondary metabolite content such as *Desmodium* and allied genera. However, limited reliability of RAPD molecular markers should be improved by increasing the number of RAPD fragments. As no correlation between the pattern of genetic clustering and geographic distance or passport data was detected, further data analyses will be necessary in order to relate genetic diversity to greater geographic distance. Increasing emphasis is being placed on empirical data of genetic diversity to assess and manage genetic resources in a sustainable way. Therefore this study may contribute to provide germplasm conservationists with data in order to facilitate conservation efforts.

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