Diversity in the tropical multipurpose shrub legumes *Cratylia argentea* (Desv.) O. Kuntze and *Flemingia macrophylla* (Willd.) Merrill

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Chapter 1

Introduction

A primary problem throughout the tropical world is increasing population pressure, resulting in the destruction of ecosystems, including the loss of biodiversity and genetic erosion (El-Swaify 1991; Humphreys 1994; Hodgkin 1997; Webster and Wilson 1999). Expansion of agricultural areas, deforestation and overgrazing can lead to soil erosion and soil fertility depletion and contribute to the destruction of natural habitats.

The role of legumes

Legumes, the world's second most important plant family used by man after the grasses, are found in temperate and tropical environments from sea level up to 4,000 m, ranging from arid to humid conditions. They offer many potential benefits to agriculture, particularly in the tropics, because of their ability to enhance soil fertility and their contribution to erosion and weed control, livestock nutrition and fallow improvement (NAS 1979; Gutteridge and Shelton 1994; Giller and Wilson 2001; Peters *et al.* 2001; Shelton 2001).

This is firstly due to the ability of most species to fix atmospheric nitrogen (N) which results in a high N content in aboveground biomass and subsequent enhancement of soil biological activity (soil structure, aeration, water balance) and soil fertility. The soil improving effect is likely to be particularly strong in low-fertility soils that cover vast areas of the subhumid and humid tropics (Oxisols and Ultisols). These soils are mostly acid and highly Al-saturated and, together with eroded, degraded soils, form part of those marginal lands where agricultural production is often severely constrained. Secondly, many legume species, in particular shrubs and trees, have a deep-reaching tap-root system which enables them to tolerate long dry spells and act as nutrient and water pumps (Humphreys 1994; Schultze-Kraft and Peters 1997).

Multipurpose tree and shrub (MPT) legumes

Multipurpose tree and shrub (MPT) legumes provide various services or products at the same time (Gutteridge and Shelton 1994; Schultze-Kraft and Peters 1997; Shelton 2000). They can be used, for example, in alley cropping systems and planted fallows, as mulch or green manure, as soil cover and for weed control, as erosion barrier, windbreak and living fences, as shade trees and for fuelwood and/or for cut-and-carry forage or feed supplementation in the dry season. In the latter case MPT have the potential to substantially contribute to improved tropical livestock production, mainly in the dry season, by providing high-quality forage, be it in forage-only (= livestock-only) production systems or in integrated (such as crop-livestock) systems (Fässler and Lascano 1995; Barnes 1997; Preston *et al.* 2000). MPT are also a

significant element of resource protection in integrated and sustainable production systems and provide direct ecological and economic benefits (feed production, soil enhancement and erosion control).

Legume research carried out by the International Centre of Tropical Agriculture (CIAT) in Cali, Colombia, concentrates on MPT with tolerance of extended dry seasons to develop legume-based, soil fertility restoring technologies for sustainable agricultural production. Tropical shrub legumes of high quality for better soils are readily available, but species adapted to acid, infertile soils are scarce (Schultze-Kraft 1996; Shelton 2001). On the other hand, the land belonging to smallholder farmers is largely located in areas with such soils. Of particular concern are the hillsides of Latin America where infertile, acid soils are combined with steep slopes compounding the problem of resource degradation.

Genetic erosion

Agricultural mismanagement, deforestation and overgrazing on the one hand, and the displacement of traditional sustainable farming systems such as shifting cultivation with restorative fallows by monoculture production systems, on the other hand, have led to genetic erosion. This affects wild plants as well as traditional cultivars and landraces. Many wild populations and species, whose potential uses are until now mostly totally unknown, have probably already become extinct. Furthermore, the number of traditional, genetically usually highly variable cultivars has decreased significantly while at the same time the use of a small number of uniform crop cultivars has increased (Tanksley and McCouch 1997).

Particularly in wild relatives of crop species and in locally adapted landraces, there is clear evidence for a dramatic amount of genetic erosion (Hodgkin 1997). Local landraces, wild relatives of crop species and, especially, wild species contain many useful characters such as adaptations, resistances and/or valuable chemical components and are therefore considered to be the raw material for breeding programmes aiming to meet the nutritional needs of future generations (Hodgkin 1997; Tanksley and McCouch 1997). The loss of genetic diversity is, however, not only an economic problem but in the long run also an environmental problem, due to direct and indirect benefits from ecosystem functions such as climate and water regulation, pollination, biological control of pests, generation and renewal of soil fertility, and food production (Heywood and Watson 1995).

Potential forage plants in the tropics are also affected by genetic erosion. For example, in central Brazil recent searches for *Arachis pintoi* Krapov. & W.C. Gregory and *Centrosema acutifolium* Benth. germplasm at the well-documented collection sites of their respective botanical type specimens resulted unsuccessful (Schultze-Kraft *et al.* 1993).

Plant genetic resources (PGR) and their conservation

Different strategies have been developed to conserve the genetic diversity of crop species and their wild relatives for future selection and breeding programmes: *In situ* conservation of plant genetic resources (PGR) is based on the maintenance of plant populations in the habitats where they occur naturally and have evolved. This can refer to specific locations or whole (agro)ecosystems and includes also the "on farm" conservation of landraces (UNCED 1992; Brush 1995). While *in situ* conservation allows for evolutionary processes to continue (Williams 1991), *ex situ* conservation of PGR takes place outside the natural habitat, mainly in genebanks, without changes in the genetic constitution of the germplasm stored (Frankel and Soulé 1981).

The implementation of an international forum addressing plant genetic diversity conservation by the Food and Agriculture Organization of the United Nations (FAO) in the 1960s, followed by the creation of the International Board for Plant Genetic Resources (IBPGR; now International Plant Genetic Resources Institute, IPGRI, Rome, Italy) resulted in the 1970s in the establishment of a number of genebanks and the application of the *ex situ* conservation approach for plant genetic resources worldwide (Plucknett *et al.* 1987). Today, more than 700 documented seed collections worldwide hold an estimated 6 million accessions stored as seeds, including many wild species and/or varieties (Engelmann and Engels 2002).

Nevertheless, total species diversity is still very inadequately represented in germplasm collections. For example, out of 13,000 described tropical legume species only 1,200 - 1,500 are maintained in tropical germplasm collections (Schultze-Kraft *et al.* 1993).

Furthermore, there are a number of problems associated with *ex situ* conservation. Most of them are due to financial, technical and time limitations, e.g., the periodic need to regenerate seed samples because of declining viability, restricted storage and refrigeration facilities, seed multiplication, and identification of duplicates (Breese 1989; Rao 1991). Another problematic issue is the use of germplasm collections regarding evaluation, selection and breeding, where the size and heterogeneous structure of collections often hinder their efficient management and utilization (Brown 1989a; Marshall 1989; Hintum *et al.* 2000).

This is particularly true for the management of collections of perennial tropical legumes. Although these collections usually do not comprise more than 50 accessions per species (Barco *et al.* 2002; SINGER 2005), due to wild species characteristics their management is complicated and requires expensive and time-consuming seed-regeneration protocols.

Assessment of genetic diversity

The assessment of genetic diversity of tropical wild legumes has hitherto consisted mainly of morphological, physiological and agronomic germplasm characterization (e.g., Schultze-Kraft and Benavides 1988; Gutteridge 1990; Thomson *et al.* 1997; Keller-Grein *et al.* 2000). Most of these traits, however, are influenced by the environment which may cause true genetic variability to be masked. In change, genetic analysis by molecular markers reflects the actual genetic distance among species, populations or individuals, and reveals patterns of evolutionary relationships. Molecular markers are therefore considered as a useful tool for characterizing the genetic diversity of germplasm collections (Vicente 2002; Ortiz and Engels 2004).

The use of molecular markers has been proposed to verify genetic diversity assessed by phenotypic characterization or as alternative procedure alone (Hintum 1994; Schoen and Brown 1995). Other authors recommend the integrated use of morphological, agronomic, biochemical, and molecular evaluations of genetic resources because the different types of traits provide complementary information (Singh *et al.* 1991; Diwan *et al.* 1994; Gepts 1995). There are several molecular marker techniques such as microsatellites, restriction fragment length polymorphisms (RFLP), random amplified polymorphic DNA (RAPD), and amplified fragment length polymorphisms (AFLP) (Karp *et al.* 1997). The different genetic markers vary with respect to important features such as genomic distribution, level of polymorphism detected, locus specificity, reproducibility, as well as technical and financial requirements. The choice of the most appropriate technique depends on given pre-conditions such as technical and financial facilities, know-how, time constraints, presumed level of polymorphism and the specific objective (Parker *et al.* 1998; Vicente 2002; Richards 2004).

Research topic

In studies conducted by CIAT with various multipurpose tree and shrub legumes, *Cratylia argentea* (Desv.) O. Kuntze and *Flemingia macrophylla* (Willd.) Merrill had been identified as very promising (Schultze-Kraft 1996). Both species are drought-tolerant, multipurpose shrub legumes well adapted to low-fertility, acid soils of the sub-humid and humid tropics, and are especially suited for low-input smallholder production systems. They enhance soil fertility and can be used for a number of purposes such as dry season forage supplementation, live soil cover or mulch, erosion barrier hedges, shade-providing shrubs in young coffee and cocoa plantations, and others. Of both species, fairly comprehensive germplasm collections, assembled from the wild-legume flora mainly in Brazil (*C. argentea*) and Southeast Asia (*F. macrophylla*) are available. Knowledge about the extent of genetic diversity within these collections is, however, very limited.

Cratylia argentea is already increasingly adopted and utilized as forage species, particularly in the seasonally dry hillsides of Central America. Research and development are, however, so far based on only a few accessions. The potential utilization of *F. macrophylla* which is well known in Southeast Asia, is so far limited by poor forage quality and acceptability of the few evaluated accessions.

Therefore, a collaborative project between the University of Hohenheim, Germany, and CIAT, Colombia, was carried out with the following objectives:

- Firstly, to describe the diversity in the collections of *Cratylia argentea* and *Flemingia macrophylla* based on conventional germplasm characterization and evaluation procedures (morphological and agronomic traits, forage quality parameters) and by molecular markers;
- Secondly, to identify superior genotypes;
- Thirdly – based on the aforementioned results – to identify core collections based on similarity regarding
  a) germplasm origin information, hypothesizing that geographic distances and environmental differences are related to genetic diversity,
  b) morphological and phenological characterization,
  c) agronomic and forage quality evaluation, and
  d) molecular markers,
  using *F. macrophylla* as example;
- and fourthly to validate their representativeness and compare them, taking into consideration the practical implications (time and cost efficiency) of each of the aforementioned approaches for the particular case of small collections of perennial wild tropical legumes.

Selected accessions from the work will be incorporated in work with farmers in CIAT-research sites in Central America and distributed to partners. Beyond the immediate application of these two multi-purpose species for farmer utilization, the results of the use and comparison of different approaches to assess genetic diversity will probably also be applicable to other species and may thus help to improve germplasm management of tropical non-grain (i.e., multipurpose, forage) legumes in general.

The present volume comprises a series of papers resulting from the above-mentioned project. Chapter 2 describes the variability of a collection of 38 *Cratylia argentea* accessions with respect to phenological, agronomic and forage quality traits, and identifies accessions superior to the commercial cultivar (see also Andersson *et al.* 2006a). In Chapter 3, the genetic diversity in a collection of 47 *C. argentea* accessions is assessed using random amplified

polymorphic DNA (RAPD) molecular markers and compared to the results of conventional germplasm characterization (see also Andersson *et al.* 2006b).

Chapter 4 presents the results of a morphological and phenological characterization of *F. macrophylla*, identifying four different morphotypes (see also Andersson *et al.* 2006c). Chapter 5 reports on an agronomic evaluation and a preliminary palatability trial of the *F. macrophylla* world collection (see also Andersson *et al.* 2006c), while in Chapter 6 the forage quality, tannin concentration and composition of a selected subset of 25 *F. macrophylla* accessions of contrasting quality are studied (see also Andersson *et al.* 2006d). Chapter 7 presents the results of a molecular markers (RAPD) analysis, assessing the extent and structure of genetic diversity in the *F. macrophylla* collection (see also Andersson *et al.* 2006e).

In Chapter 8, different approaches (geographic origin, morphological and phenological traits, agronomic and forage quality traits, molecular markers) for creating core collections are compared and verified, taking *F. macrophylla* as an example. The practical implications of each of the strategies are discussed with particular consideration of small collections of perennial wild tropical legumes.

Chapter 2

Phenological, agronomic and forage quality diversity among germplasm accessions of the tropical legume shrub *Cratylia argentea*

Abstract

Cratylia argentea (Desv.) O. Kuntze is a drought tolerant tropical shrub legume which can help to ensure continuity of forage supply in smallholder systems either through direct grazing or as a cut-and-carry plant for fresh foliage or silage. A collection of 38 accessions was characterized agronomically and nutritionally. High diversity was detected among accessions. Time to flowering ranged from 217-329 days after transplanting seedlings to the field and from 129-202 days after cutting. Flowering is probably induced by reduction of day length. Seed production was high and variable. DM production ranged from 190-382 g plant^{-1} in the rainy and from 124-262 g plant^{-1} in the dry season, IVDMD from 589-690 g kg^{-1}, CP content from 184-237 g kg^{-1} and fibre content from 403-528 g kg^{-1} (NDF), 240-335 g kg^{-1} (ADF), and 9-13 g kg^{-1} (N-ADF). Accessions CIAT 18674 and CIAT 22406 were identified as promising for further studies; they were superior to the commercial cultivar in terms of DM 44production, particularly in the dry season. Further research is required to determine the prevailing reproduction strategy of *C. argentea* and to quantify outcrossing-rates. Multilocational trials with a selected set of accessions should be conducted under different environmental conditions.

Keywords: agronomic evaluation, *Cratylia argentea*, dry season feed, forage legume, forage quality, multipurpose, phenological characterization, shrub legume

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Introduction

Livestock productivity in the tropics is severely affected in the dry season by low availability and quality of fodder (NAS 1979; Ranjhan 1986; Enríquez Q. *et al.* 2003). High-protein legumes, in particular shrub species, can contribute to improved productivity of livestock during dry seasons and at the same time maintain and even restore soil fertility (Brewbaker 1986; Schultze-Kraft and Peters 1997; Peters *et al.* 2001; Shelton 2001). *Cratylia argentea* (Desv.) O. Kuntze (syn. *C. floribunda* Benth., *Dioclea argentea* Desv.) is a shrub legume which was selected as promising for dry-season supplementation, particularly in regions with acid soils and extended dry seasons (Argel and Maass 1995; Argel and Lascano 1998; Peters and Schultze-Kraft 2002).

Cratylia argentea belongs to the family Leguminosae, subfamily Papilionoideae, and is the most widely distributed of the five species in the genus (Queiroz and Coradin 1996; Pizarro *et al.* 1997). The leafy shrub usually reaches between 1.5 and 3 m, but there are trees up to 10 m tall. It is found in a broad range of habitats from Western Peru to the state of Ceará in Brazil, and is well adapted to acid soils of low to medium fertility and altitudes up to 1200 m asl (Xavier *et al.* 1995; Maass 1996; Schultze-Kraft 1996; Peters and Schultze-Kraft 2002). Its nutritive value is higher than that of most other shrub legumes adapted to acid soils. Plants contain only trace amounts of tannins (Lascano 1996; Shelton 2001). It has excellent regrowth capacity after cutting and can be used as soil cover, mulch and green manure.

The species is very drought tolerant and remains green and productive during dry periods of up to seven months. It grows best on well-drained soils; water logging should be avoided (Argel and Lascano 1998). Seed production is abundant, but initial growth in the establishment phase is slow, especially when soil pH is higher than 5.5. The chromosome number of *C. argentea* is $2n = 22$ (Queiroz 1991), but it is unclear whether the reproduction system of *C. argentea* is allogamous or autogamous. Queiroz *et al.* (1997) reported the occurrence of outcrossing in a preliminary study and suggested a mixed mating system.

The utility of *C. argentea* in production systems is as a protein source, particularly during the dry season, both in cut-and-carry (fresh fodder and silage) and grazing systems (Schultze-Kraft and Peters 1997; Argel and Lascano 1998; Jiménez *et al.* 2001; Holmann *et al.* 2002; Lascano *et al.* 2002). Though its potential as a forage plant had been recognized as early as the 1940s (Otero 1961), until about 25 years ago the species was essentially unknown to the scientific community and started to attract wider scientific attention only recently. Although the introduction of *C. argentea* into production systems is fairly recent, farmers in some Latin American countries are already growing it, particularly in Costa Rica and Colombia. A small collection of 11 accessions has been evaluated in different environments (various authors in Pizarro and Coradin 1996; Schultze-Kraft 1996), and as a result, the mixture of accessions

CIAT 18516 and CIAT 18668 was released in Costa Rica as cv. Veraniega (Argel *et al.* 2001) and in Colombia as cv. Veranera (Lascano *et al.* 2002). Today, germplasm collections with about 50 accessions are held at the Centro Internacional de Agricultura Tropical (CIAT), Cali, Colombia, and at Embrapa Recursos Genéticos e Biotecnologia, Brasília, Brazil.

The objective of this study was to assess the diversity of the available collection of *C. argentea* germplasm in terms of phenological, agronomic and forage quality traits, and to identify superior accessions.

Materials and methods

Evaluation site characteristics

The collection available for this study comprised 38 accessions from Brazil. Accession CIAT 18668, one of the two accessions that compose the cultivar Veraniega in Costa Rica (Veranera in Colombia) was chosen as control for comparison.

The trial was established at the Quilichao Experimental Station of the International Centre for Tropical Agriculture (CIAT) in the Cauca department, Colombia (03°06' N, 76°31' W; 990 m asl). Annual rainfall is 1800 mm, distributed in a bimodal pattern with two generally well pronounced rainy seasons from March to June and from September to December. Rainfall during the establishment period (April 1999–April 2000) was 1928 mm, and 2107, 1345 and 1265 mm during the three subsequent experiment years (Figure 2.1). In one of the harvest months at the end of a dry season (September 2001), however, rainfall was up to 100 mm which cannot be classified as an actual “dry” period. Therefore, from now on when speaking of rainy and dry season it is actually referred to periods of maximum and minimum rainfall.

The soil is an Ultisol (pH = 5.3) with 76% Al saturation, medium P (6 ppm Bray II) and high organic matter content (7.4%). Seeds were sown into Jiffy pots in the greenhouse and inoculated with *Bradyrhizobium* strain CIAT 3561. After six weeks, the seedlings were transplanted to the field. *Cratylia argentea* responds well to inoculation with cowpea-type *Rhizobia* strains that are native to most tropical soils (Neves and Rumjanek 1997; Bala *et al.* 2003). However, for acid and infertile soils inoculation with *Bradyrhizobium* strains CIAT 3561 and 3564 is recommended to assure that the lack of inoculum is not a limiting factor to plant establishment (Lascano *et al.* 2002).

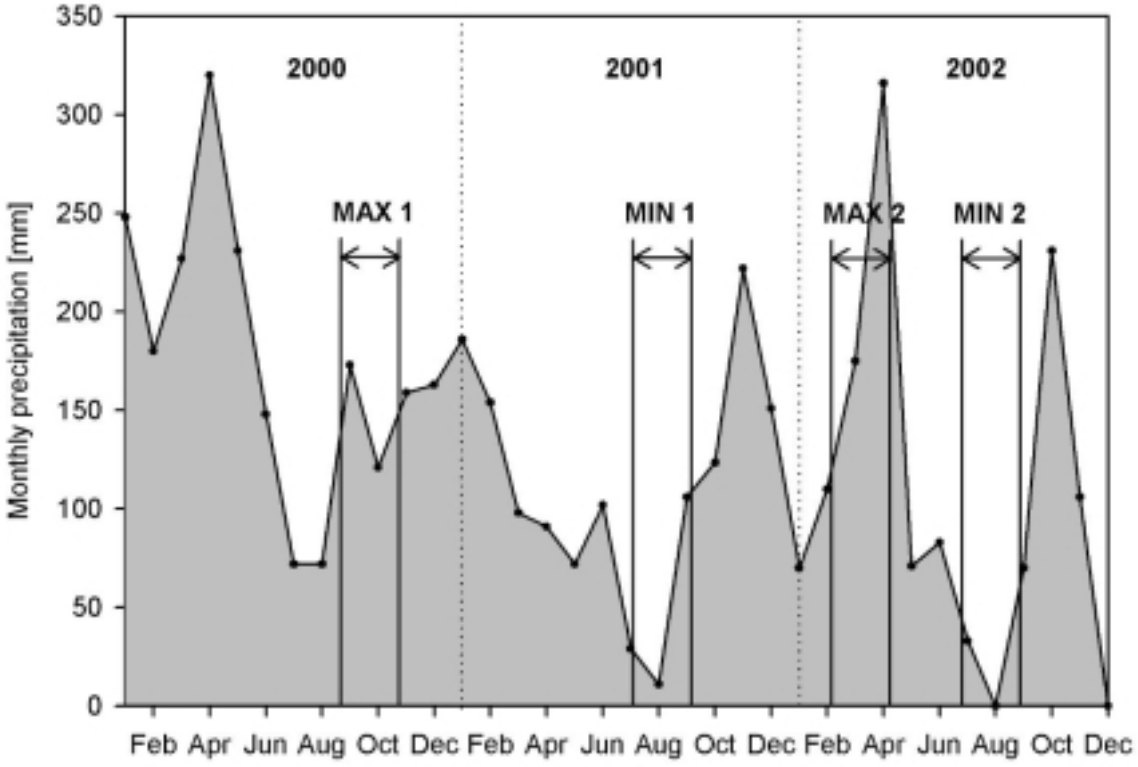

Figure 2.1 Monthly precipitation and rainy and dry seasons at the CIAT-Quilichao Experimental Station, Colombia, during the three study years. Evaluation cuts were conducted at the end of each season

For the assessment of phenological traits, unreplicated 4-plant single-row plots were established, with a distance of 1 m between plants. Results of the four individual plants were averaged. For the agronomic and forage quality evaluation, a Randomised Complete Block design with three replications was used, each arranged as 7-plant single-row plots with a distance of 1 m between plants and 1.5 m between rows. Fertilizer was applied to single plants four weeks after transplanting at a rate of 40, 50, 20 and 0.5 kg ha^{-1} of P, K, Mg and Mo, respectively. In the phenological characterization plots, a standardization cut was performed 14 months after transplanting at a height of 70 cm, and in the agronomic evaluation plots after 12 months, to correct for eventual differences in plant development and assure uniform growth.

Phenological characterization

The collection was characterized assessing time to 50% flowering, number of pods per plant, seed weight and seed yield. The values presented are the means of measurements or

observations of four plants per accession. Time to 50% flowering was recorded after transplanting as well as after the standardization cut (after 14 months). Fifty percent flowering was defined as 50% of the buds of a single plant flowering. The total number of pods and cumulative seed yield (g) per plant (handpicking of all ripe pods twice a week) were recorded, considering all pods and seeds, respectively, that were produced until the standardization cut. The weight of randomly sampled 100 seeds (three replications) was determined and subsequently 1000-seed weight calculated.

Agronomic evaluation

During the establishment period, evaluations were conducted at 8, 12, 16 and 20 weeks after transplanting of the seedlings to the field, measuring plant height and plant diameter, rating plant vigour, and visually assessing the incidence of pests, diseases and mineral deficiencies.

A standardization cut was performed 12 months after planting at a height of 70 cm. In previous evaluations at the same site, *C. argentea* has shown good performance when cut at 30 cm (Maass 1996). However, in view of the findings of Lobo & Acuña (2001), who observed a tendency of increased DM production with increased cutting height (90cm>60cm>30cm), in the present study a cutting height of 70 cm was chosen.

C. argentea can usually be cut after an establishment phase of six to eight months (Lascano *et al.* 2002), but in this experiment plants suffered a delay after a severe nematode attack which was controlled by applying a nematicide. Plants were then cut back and an additional standard fertilization dose was applied to ensure good and uniform regrowth. All plants recovered well and showed good regrowth, and there were only minor incidences of pests and no visible incidences of diseases or mineral deficiencies during the subsequent evaluation period. Nematode problems (probably *Oryctanthus occidentalis* Eichl., family Iorenthaceae) in *C. argentea* had been observed earlier at Quilichao (Maass 1996), but no significant pest or disease problems have been reported for the species at more than 40 other locations in different environments (various authors in Pizarro and Coradin 1996; Lascano *et al.* 2002), indicating that the nematode problem is probably location specific.

After the standardization cut, plants were cut at 8-week intervals during 36 months. Dry matter (DM) production (g plant^{-1}) of the three central plants was measured during two rainy and two dry seasons, separating edible (leaf and stem with <10 mm diameter) and non-edible (stem with >10 mm diameter) fractions. Samples were oven-dried at 70 °C for 72 hours, and subsequently edible, non-edible and total DM production (g plant^{-1}) were calculated. Before each evaluation cut, regrowth capacity (number of regrowth shoots per plant), and incidence of pests, diseases and mineral deficiencies were assessed.

Forage quality analysis

Forage quality was analysed in two dry seasons and one rainy season. Leaves were harvested when plants were cut for the agronomic evaluations. Samples were freeze-dried and ground to pass a 1-mm screen in a Wiley mill. *In vitro* dry matter digestibility (IVDMD) was estimated following the two-stage technique (Tilley and Terry 1963), modified by Harris (1970). Total nitrogen was determined by the Kjeldahl procedure (AOAC 2003) and multiplied by 6.25 to obtain an estimate of crude protein (CP). Accessions were analysed for neutral and acid detergent fibre (NDF and ADF) and acid detergent fibre-bound nitrogen (N-ADF) (Soest *et al.* 1991). Sodium sulphite was added to the neutral detergent solution to remove traces of tannin-protein complexes (Robbins *et al.* 1987).

Statistical analysis

Phenological, agronomic and forage quality data were subjected to Principal Component Analysis (PCA, Jolliffe 2002). Correlations were calculated and when the correlation coefficient (r) between two variables was greater than 0.7, then only one of these was included in the subsequent cluster analysis (Ward's hierarchic clustering method, Ward 1963). After the nematode attack in the establishment phase of the agronomic evaluation, the incidence of pests, diseases and mineral deficiencies was negligible and therefore excluded from the cluster analysis. Data on regrowth capacity, DM production, IVDMD, CP, ADF, NDF and N-ADF were subjected to analysis of variance (GLM-ANOVA procedure). The effects of replication (n = 3), genotype (nciat) and season (dry, wet) were tested on the traits, using the simple, fixed ANOVA model : $Yijk = \mu + Ri + Gj + RG(ij) + Sk + SR(ki) + SG(kj) + eijk$, in which Yijk is the observation, μ is the general mean, Ri is the effect of replication, Gj the effect of genotype, Sk the effect of season, RG(ij) is replication x genotype, SR(ki) is season x replication, SG(kj) is season x genotype interaction, and eij is the error term. Means were separated at $P < 0.05$ by the Ryan-Einot-Gabriel-Welsch Multiple Range test. All analyses were performed using the SAS program package version 8.2 (SAS Institute Inc. 1999).

Results

Plant establishment

Plant establishment was slow during the first months and manual weeding was required. After eight weeks, plant height ranged from 16 to 22 cm (average 19 cm) and plant diameter from 19 to 28 cm (average 24 cm). Plant vigour was low (average 2.5), and except for the site-specific nematode attack which showed no differences among accessions, there was virtually no incidence of pests, diseases or mineral deficiencies (averages of 1.4, 0.0 and 0.0, respectively). All 38 *Cratylia argentea* accessions adapted well to the experimental site and no accession was lost.

Flowering and seeding

Large variability was detected among accessions for phenological traits (Table 2.1). Time to 50% flowering ranged from 217 to 329 days (average 275 days) after transplanting, and was conspicuously reduced (average 172 days) after cutting. In both cases, the principal flowering peak was in December, i.e. some weeks after the dry season (July to September). The number of pods per plant ranged from 11 to 387 (average 117) and seed yield from 18 to 757 g plant^{-1} (average 179 g plant^{-1}). The large variability was due to the fact that some plants flowered with very low inflorescence numbers and some did not flower at all. Seed weight ranged from 220 to 383 g 1000-seeds^{-1} (average 281 g 1000-seeds^{-1}).

Agronomic evaluation and forage quality

After the standardization cut, large variability among accessions was detected in terms of regrowth capacity, DM production, IVDMD and CP content (Table 2.2). Only minor, negligible incidence of pests and no incidence of diseases or mineral deficiencies were detected during the subsequent evaluation periods. The number of regrowth shoots varied between 13 and 29 shoots per plant. Dry matter production ranged from 190 to 382 g plant^{-1} (average 265 g plant^{-1}) in the rainy season, and from 124 to 262 g plant^{-1} (average 192 g plant^{-1}) in the dry season. Of the total DM production, 100% was edible DM since no stems with a diameter of more than 10 mm were developed within eight weeks after cutting. IVDMD varied from 589 to 690 g kg^{-1} and CP content from 184 to 237 g kg^{-1}. NDF ranged from 403 to 528 g kg^{-1}, ADF from 240 to 335 g kg^{-1} and N-ADF from 9 to 13 g kg^{-1} (Table 2.2).

Genotypes differed ($P < 0.01$) for regrowth capacity, DM production, IVDMD, CP and fibre content. Season significantly ($P < 0.01$) affected DM production and ADF. Total DM production in the dry season averaged 73% of that during the rainy season. Accessions CIAT 22373, CIAT 22376, CIAT 22382, CIAT 22393, CIAT 22407 and CIAT 22409 had DM yields in the dry season which were more than 80% of those in the rainy season. A genotype x season interaction ($P < 0.01$) was detected for IVDMD (Table 2.3). All accessions were stable across seasons, with the exception of accessions CIAT 18676, CIAT 22387 and CIAT 22390 which had significantly higher (LSD 27.63, $P < 0.05$) IVDMD values in the dry than in the rainy season.

Agronomic groups

The cluster analysis dendrogram was truncated at the 10-group level, explaining 58% of total variation (Figure 2.2). Dry matter production, IVDMD, earliness, and seed production were the main agronomic characteristics used to separate the ten groups (Table 2.4).

Table 2.1 Flowering behaviour and seed production of 38 *Cratylia argentea* accessions at the CIAT-Quilichao Experimental Station, Colombia

	after transplanting to the field*				after cutting
Accession (CIAT No.)	days to 50% flower	pods per plant	1000-seed weight	seed yield	days to 50% flower
	(no.)	(no.)	(g)	(g plant^{-1})	(no.)
18516	329	11	383.3	18	183
18667	313	229	330.7	387	180
18671	251	387	263.7	757	176
18672	301	142	275.7	146	175
18674	307	81	331.1	153	202
18675	305	123	249.8	171	192
18676	297	159	322.3	278	177
18957	253	288	257.4	426	163
22373	288	73	286.7	93	161
22374	291	66	285.3	111	170
22375	289	113	357.9	255	174
22376	276	112	260.2	178	193
22378	272	22	266.7	40	186
22379	270	73	323.1	114	179
22380	285	101	246.8	126	190
22381	304	85	266.2	117	192
22382	255	77	262.8	126	175
22383	298	29	264.1	36	173
22384	270	92	323.7	165	185
22386	304	99	241.4	136	194
22387	221	138	281.0	254	135
22390	217	93	265.3	163	140
22391	322	60	331.9	101	183
22392	300	88	289.1	152	174
22393	223	131	257.3	159	143
22394	232	100	273.8	175	130
22396	283	120	288.0	179	182
22399	236	124	257.2	151	171
22400	285	164	219.7	158	173
22404	280	182	247.2	211	170
22406	261	126	283.6	152	130
22407	267	103	247.4	126	157
22408	265	130	280.5	153	176
22409	260	85	250.9	97	185
22410	287	116	240.8	181	199
22411	240	138	268.0	233	129
22412	238	141	298.4	219	153
Control					
18668	276	51	301.7	110	183
Mean	275	117	281.1	179	172
SEM	4.7	11.2	5.74	20.4	3.2

* average of four plants per accession

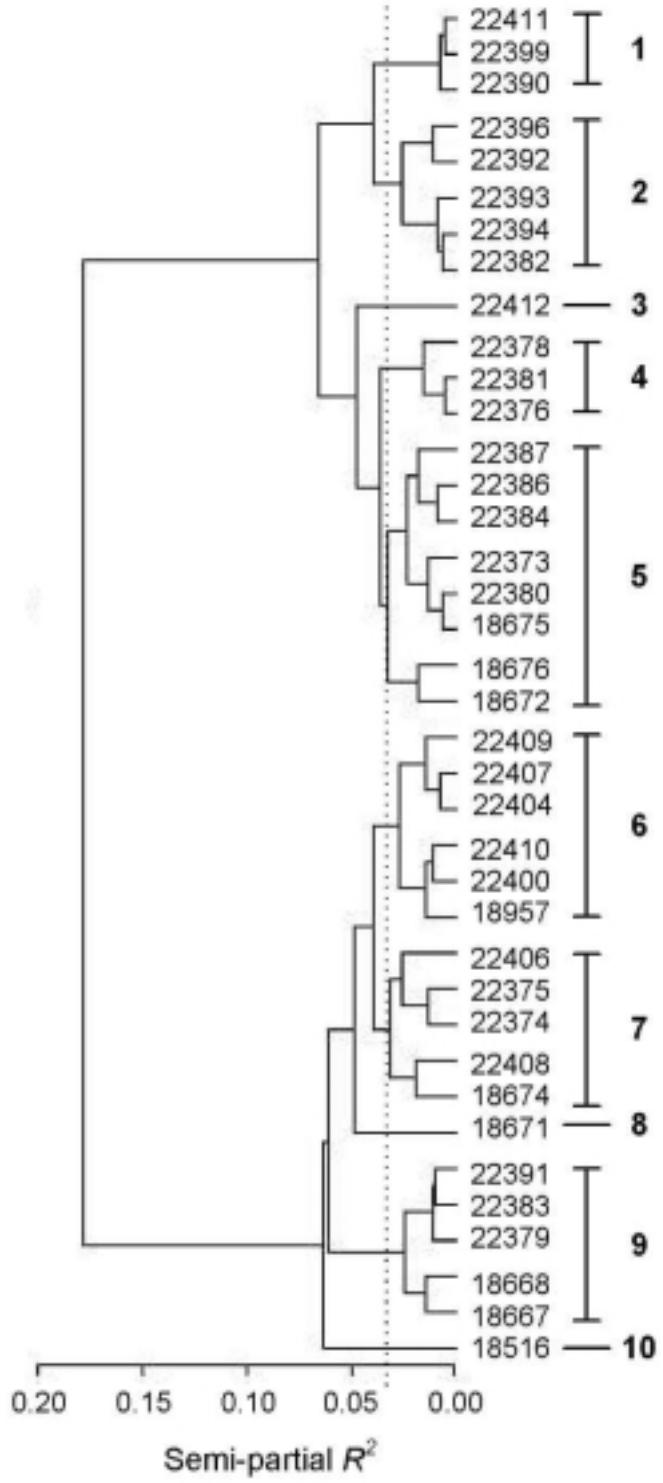

Figure 2.2 Dendrogram classification of 38 accessions of *Cratylia argentea*, by principal component and cluster analysis according to phenological, agronomic and forage quality data, truncated at the 10-group level (dotted line)

Table 2.2 Regrowth capacity, dry matter (DM) yield, *in vitro* dry matter digestibility (IVDMD), crude protein (CP) and fibre (neutral detergent fibre, NDF; acid detergent fibre, ADF; N-ADF) content of 38 *Cratylia argentea* accessions at the CIAT-Quilichao Experimental Station, Colombia, in the rainy and dry season

Accession (CIAT No.)	Regrowth*		DM yield		IVDMD		CP		NDF		ADF		N-ADF	
	rainy	dry	rainy	dry	rainy	dry	rainy	dry	rainy	dry	rainy	dry	rainy	dry
	(no. shoots plant^{-1})		(g plant^{-1})		(g kg^{-1})		(g kg^{-1})		(g kg^{-1})		(g kg^{-1})		(g kg^{-1})	
18516	26	20	306	204	642	666	209	235	411	447	251	277	11	11
18667	23	19	264	182	643	644	210	225	439	430	257	289	10	9
18671	23	21	278	179	630	670	194	226	416	450	256	289	10	9
18672	19	16	233	139	609	626	207	215	453	454	273	308	11	10
18674	29	23	382	244	652	651	205	230	426	455	258	305	10	10
18675	19	18	281	195	624	652	193	217	478	459	286	307	10	9
18676	20	18	282	199	589	652	192	221	441	430	266	290	13	11
18957	22	20	303	214	624	642	209	220	444	460	282	294	11	10
22373	21	20	247	202	631	647	208	217	482	475	273	322	11	11
22374	23	18	302	208	664	665	203	222	438	512	251	309	11	10
22375	21	20	312	241	654	656	215	233	432	528	262	302	12	11
22376	15	16	207	171	636	638	199	206	441	496	263	311	10	9
22378	18	14	190	149	609	620	206	211	472	467	277	324	11	10
22379	22	18	284	202	630	649	205	217	425	442	257	308	10	9
22380	16	15	259	170	619	648	205	224	460	485	282	333	9	10
22381	18	13	222	138	645	634	202	211	440	499	265	335	10	10
22382	17	16	251	217	632	655	210	224	444	428	272	319	10	10
22383	19	16	245	179	623	640	198	220	429	441	276	308	9	9
22384	13	13	263	150	634	664	202	211	447	455	290	308	10	9
22386	16	14	233	168	637	672	195	204	427	441	276	312	10	9
22387	18	14	233	165	590	670	192	209	449	475	273	325	10	10
22390	18	15	211	153	619	683	194	212	439	464	268	289	12	10
22391	21	19	276	169	627	675	196	219	430	447	282	311	9	9
22392	19	17	218	124	627	662	222	226	472	466	274	322	10	10
22393	23	21	250	210	629	659	220	226	467	446	260	313	11	9
22394	19	17	219	160	637	667	217	226	438	446	260	291	11	10
22396	14	14	198	146	642	652	219	235	459	469	274	304	10	10

22399	20	15	196	149	658	679	208	216	436	462	250	290	12	10
22400	23	20	274	200	636	632	209	221	434	445	269	308	10	11
22404	18	17	276	220	679	663	212	232	445	450	244	282	11	10
22406	27	24	354	259	636	664	213	218	484	509	277	335	10	10
22407	21	21	285	243	665	662	210	218	453	455	260	306	11	10
22408	21	18	325	245	690	670	213	221	413	443	240	291	9	10
22409	22	21	311	262	662	676	218	237	462	449	273	298	10	10
22410	20	24	303	237	633	643	197	226	439	465	257	310	12	10
22411	18	17	245	171	650	674	208	209	455	472	261	302	11	10
22412	15	16	299	231	641	649	184	210	486	501	271	317	10	12
Control														
18668	24	22	239	191	642	652	211	228	403	429	255	295	11	10
Range	13-29	13-24	190-382	124-262	589-690	620-683	184-222	204-237	403-486	428-528	240-290	277-335	9-13	9-12
Mean	20	18	265	192	636	656	206	220	445	462	266	306	10	10
SEM	0.6	0.5	20.4	10.8	3.3	4.2	1.4	1.7	20.5	24.4	11.8	14.4	0.8	0.6

* Regrowth of 8 weeks; data from two evaluation cuts per season except for IVDMD, CP and fibre content in the rainy season (one evaluation cut).
SEM, standard error of the mean

Table 2.3 *Cratylia argentea* accessions stable across seasons and accessions showing genotype x season interaction for the forage quality trait *in vitro* dry matter digestibility (IVDMD)

	CIAT No.	IVDMD (g/kg)	CIAT No.	IVDMD (g/kg)
stable behaviour across seasons	18516	654	22386	655
	18667	644	22391	651
	18671	650	22392	645
	18672	618	22393	644
	18674	652	22394	652
	18675	638	22396	647
	18957	633	22399	669
	22373	639	22400	634
	22374	665	22404	671
	22375	655	22406	650
	22376	637	22407	664
	22378	615	22408	680
	22379	640	22409	669
	22380	634	22410	638
	22381	640	22411	662
	22382	644	22412	645
	22383	632	18668 (Control)	647
	22384	649		
		LSD ($P < 0.05$)	24.06	
genotype x season interaction		CIAT No.	Rainy / dry	
		18676	589 / 652	
		22387	590 / 670	
		22390	619 / 683	
		LSD ($P < 0.05$)	27.63	

Clustering divided the accessions into two group complexes of contrasting DM production and IVDMD: Groups 1 to 5 generally have only low to moderate DM yields and moderate to high IVDMD in the dry season, whereas groups 6 to 10 have moderate to high DM production combined with consistently high IVDMD in the dry season. Within the latter, groups 6 and 7 are the most promising ones because they have the highest DM yields and IVDMD in the dry season. Group 6 comprises six accessions (CIAT 18957, CIAT 22400, CIAT 22404, CIAT 22407, CIAT 22409 and CIAT 22410) which have high DM production (229 g plant^{-1}) and high IVDMD (653 g kg^{-1}) in the dry season, and high seed yield (200 g plant^{-1}).

Group 7 is composed of five accessions (CIAT 18674, CIAT 22374, CIAT 22375, CIAT 22406 and CIAT 22408) with the highest mean DM production (335 g plant^{-1} in the rainy and 240 g plant^{-1} in the dry season) and IVDMD (659 and 661 g kg^{-1}, respectively) of the whole collection. Time to flowering of accessions in this group is intermediate (315 days), and seed yield moderate (162 g plant^{-1}).

Table 2.4 Identification of *Cratylia argentea* accessions of agronomic interest according to dry matter (DM) production, *in vitro* dry matter digestibility (IVDMD), earliness and seed production

Group	Agronomic characteristics*	Accession (CIAT No.)
1	low DM yields, moderate to high IVDMD, early flowering, moderate seed yield	22390, 22399, 22411
2	low to moderate DM yields, moderate to high IVDMD, early flowering, low seed yield	22382, 22392, 22393, 22394, 22396
3	high DM yields, moderate IVDMD, early flowering, high seed yield	22412
4	low DM yields, moderate IVDMD, intermediate time to flowering, low seed yield	22376, 22378, 22381
5	moderate DM yields, low to high IVDMD, intermediate time to flowering, moderate seed yield	18672, 18675, 18676, 22373, 22380, 22384, 22386, 22387
6	moderate to high DM yields, moderate to high IVDMD, intermediate time to flowering, moderate seed yield	18957, 22400, 22404, 22407, 22409, 22410
7	high DM yields, high IVDMD, intermediate time to flowering, moderate seed yield	18674, 22374, 22375, 22406, 22408
8	moderate to high DM yields, moderate to high IVDMD, early flowering, high seed yield	18671
9	moderate DM yields, moderate to high IVDMD, late flowering, low seed yield	18667, 18668, 22379, 22383, 22391
10	high DM yields, moderate to high IVDMD, late flowering, low seed yield	18516

* DM production: in the rainy season: low: <250 g plant^{-1}, moderate: 250 to 299 g plant^{-1}, high: ≥300 g plant^{-1}; in the dry season: low: <160 g plant^{-1}, moderate: 160 to 200 g plant^{-1}, high: ≥200 g plant^{-1}; IVDMD: low: <620, moderate: 620 to 649, high: ≥650 g kg^{-1}; earliness: early: <300 days, intermediate: 300 to 320 days, late flowering: >320 days; seed production: low: <160 g plant^{-1}, moderate: 160 to 200 g plant^{-1}, high: >200 g plant^{-1}

Group 7 is composed of five accessions (CIAT 18674, CIAT 22374, CIAT 22375, CIAT 22406 and CIAT 22408) with the highest mean DM production (335 g plant^{-1} in the rainy and 240 g plant^{-1} in the dry season) and IVDMD (659 and 661 g kg^{-1}, respectively) of the whole collection. Time to flowering of accessions in this group is intermediate (315 days), and seed yield moderate (162 g plant^{-1}).

Among the accessions superior to the commercial cultivar, CIAT 18674 and CIAT 22406 (both Group 7) were identified as promising for further testing because they combined high productivity (up to 382 g plant^{-1} in the rainy and up to 259 g plant^{-1} in the dry season) with high IVDMD, high CP content and moderate seed yield (Table 2.5).

Table 2.5 Dry matter (DM) production, *in vitro* DM digestibility (IVDMD), crude protein (CP) content, time to flowering and seed yield of the two selected promising *Cratylia argentea* accessions and the control accession CIAT 18668

Accession (CIAT No.)	rainy season			dry season			Time to flowering*	Seed production*
	DM production	IVDMD	CP	DM production	IVDMD	CP		
	(g plant^{-1})	(g kg^{-1})	(g kg^{-1})	(g plant^{-1})	(g kg^{-1})	(g kg^{-1})	(days)	(g plant^{-1})
18674	382	652	205	244	651	230	339	153
22406	354	636	213	259	664	218	293	152
18668 (Control)	239	642	211	191	652	228	308	110

Regrowth of 8 weeks. Data from two evaluation cuts per season, except for IVDMD and CP (one evaluation cut in the rainy season)
* after transplanting to the field

Discussion

Phenological and agronomic characteristics of *Cratylia argentea* differed greatly among accessions. High diversity was measured particularly for the traits earliness, seed yield, regrowth capacity, DM production and IVDMD.

Flowering and pod setting

Flowering (and thus pod setting) occurred considerably earlier after standardization than after transplanting (172 vs. 275 days, respectively), and seed production was higher (179 vs. 190 g plant^{-1}, respectively). High seed production of tropical legumes after cutting is unusual. In other perennial species, seed production is mostly reduced after cutting, e.g. in *Gliricidia sepium* (Jacq.) Walp.(Atta-Krah 1987) and *Desmodium intortum* (Mill.) Urb.(Roden *et al.* 2002). Cutting of *C. argentea* seed production plots is therefore recommended. The synchronization of flowering and pod setting is generally low, and therefore pods need to be hand-harvested continuously during 2 to 3 months (Maass 1996; Argel and Lascano 1998).

In the present study, *C. argentea* flowered at the end of the rainy season (flowering peak in December, after transplanting as well as after cutting) which is in agreement with other reports of flowering times of *C. argentea* in Colombia (Lascano *et al.* 2002). This could indicate flowering induction by water stress during the dry season as well as by reduced day length after the equinox (21st September). In the state of Minas Gerais (Brazil) in the humid tropics, however, *C. argentea* flowered at the beginning of the dry season between May and June (Xavier and Carvalho 1996), after a rainy season of five to six months. In both cases, flowering initiated within few weeks after the autumn equinox (21st September in the northern, 21st March in the southern hemisphere), thus indicating that flowering induction in *C. argentea* seems to be affected mainly by photoperiod (shorter days), and not as much by water stress.

Little is known about the mating system of *C. argentea* (self-fertilized or cross-pollinated) and more research is needed in this field. The only study investigating the mating system of *C. argentea* is that of Queiroz *et al.* (1997), who reported that *C. argentea* reproduces by both autogamy and allogamy. The presence of various potential pollinators, among them ants, bees and bumblebee species such as *Apis mellifera*, *Bombus* sp., *Centris* sp. and *Xylocopa* sp. (Hymenoptera: Anthophoridae) has been documented (Queiroz 1996; Sobrinho and Nunes 1996; Xavier and Carvalho 1996).

It is hypothesized that "tripping", a pollination mechanism where the - otherwise non-functional - sexual column is released from the keel by insects (usually bees or bumblebees), is involved. Tripping has been reported for various legumes, where it is required for successful fertilization and thus seed-setting, e.g. in *Medicago sativa* L. (McGregor 1976) and *Centrosema macrocarpum* Benth. (Escobar and Schultze-Kraft 1990). This would indicate that *C. argentea* is potentially autogamous but that a significant amount of cross-pollination may occur due to pollen transfer by visiting insects, thus suggesting a mixed mating system. This is further supported by the high genetic variability within accessions detected using Random Amplified Polymorphic DNA (RAPD) (Chapter 3).

Until more information on the genetic structure within the species is available, it is suggested that pure genotypes of *C. argentea* accessions should be maintained and germplasm banks therefore need to keep accessions in reproductive isolation. It might also be necessary to consider bagging of inflorescences or reconsider minimum separation distances between accessions of at least 300 m (A. Ciprián, CIAT Genetic Resources Unit, pers. communication 2005) during multiplication in the field.

Forage production

The average DM production (229 g plant^{-1}) was three-fold higher than reported earlier for *C. argentea* for the same location (but with lower soil fertility, Schultze-Kraft 1996), and similar to that of *Flemingia macrophylla*, another drought-tolerant multipurpose shrub legume evaluated during the same time period at the same site (Chapter 5, Andersson *et al.* 2005). The dry season DM production of *C. argentea* was high (73% of that in the rainy season). This indicates good drought tolerance of *C. argentea*. However, this may not be a true conclusion since rainfall was up to 100 mm in one of the harvest months at the end of a dry season (September 2001).

The higher productivity of *C. argentea* in this study as compared to reports from Brazil (Xavier *et al.* 1990) and Mexico (Enríquez Q. *et al.* 2003), suggests that rainfall distribution and high soil organic matter content (7.4%) favoured growth of *C. argentea*. Multilocational regional trials with a selected subset of accessions (including the two promising accessions

identified in this study) should be conducted in order to assess their adaptation under different climatic and edaphic conditions. The subset should also include accessions that have been collected in geographically distinct regions, such as the Amazon Region (e.g. CIAT 18672 from Pará and CIAT 18957 from Tocantins) or new germplasm from Bolivia, based on the hypothesis that greater geographic distance implies greater differences in genetic diversity.

Forage quality

The IVDMD values recorded in the present study (650 g kg^{-1} ± 25.9) exceed those reported earlier for *C. argentea* for the same location by Lascano (1996), Schultze-Kraft (1996) and Argel and Lascano (1998). They are also higher than values recorded by Xavier and Carvalho (1996) for Brazil and by Romero and González (2001) for Costa Rica.

The effect of season on IVDMD differed among accessions, as indicated by a genotype x season interaction ($P < 0.01$). Most accessions were stable across seasons, but accessions CIAT 22387, CIAT 18676 and CIAT 22390 had relatively higher DM digestibility in the dry than in the rainy season. However, this had little agronomic implications since their DM production in the dry season was only moderate (165, 199 and 153 g $plant^{-1}$, respectively) and only accessions with higher DM production than the control are of interest for further evaluation.

Less variation among accessions was detected in crude protein (184 to 237 g kg^{-1}) and fibre content (NDF: 403 to 528 g kg^{-1}, ADF: 240 to 335 g kg^{-1}, N-ADF: 9 to 13 g kg^{-1}). Mean values were similar to those recorded by Fässler and Lascano (1995), Wilson and Lascano (1997) and Argel and Lascano (1998) for the same location.

It had been shown earlier that the nutritive quality of *C. argentea* is similar to that of other high quality shrub legume species widely used for ruminant nutrition in the tropics, e.g. *Gliricidia sepium* (Jacq.) Walp. and *Leucaena leucocephala* (Lam.) de Wit. (Lascano 1996; Shelton 2001; Lascano *et al.* 2002), but lower than that of *Desmodium velutinum* (Willd.) DC. (Schultze-Kraft *et al.* 2005). The findings show that there is greater diversity in the *C. argentea* collection with respect to DM production than with respect to forage quality.

Conclusions

Variation in agronomic characteristics was detected in the *Cratylia argentea* germplasm collection of 38 accessions in terms of higher DM production, particularly in the dry season. Accessions CIAT 18674 and CIAT 22406 were identified as promising because they are similar to the control CIAT 18668 in terms of forage quality, but superior in terms of DM production, particularly in the dry season. The DM production of *C. argentea* was higher at Quilichao than reported from other locations possibly as a result of environmental

interactions. Therefore, multilocational trials should be conducted with a selected subset of accessions (including the two promising accessions identified in this study) to identify accessions with consistently high DM production under different climatic and edaphic conditions, as well as accessions adapted to specific niches.

Photoperiodic changes (reduced day length) were identified as the main external factor, inducing flowering in *C. argentea*. Further research is warranted in order to determine the prevailing reproduction method of the species and to quantify the rate and impact of outcrossing. This information is crucial for the development of sound germplasm management strategies and for deciding if pooling of some accessions might be justified. Meanwhile, however, accessions should be kept in reproductive isolation in order to maintain pure genotypes.

Chapter 3

Extent and structure of genetic diversity in a collection of the tropical multipurpose shrub legume *Cratylia argentea* (Desv.) O. Kuntze as revealed by RAPD markers

Abstract

The tropical multipurpose shrub legume *Cratylia argentea* (Desv.) O. Kuntze is well adapted to acid soils of low to medium fertility and has excellent drought-tolerance. Due to its high nutritive value it is particularly suited as dry-season supplementation. *C. argentea* accessions differ in geographic origin, and in morphological and agronomic characteristics. In a molecular study of a 47-accession collection, random amplified polymorphisms were effective for assessing genetic diversity. One taxonomic mismatch and five duplicate accessions were identified. Genetic diversity (H_S = 0.123) in the collection was low and genetic similarity among accessions high (GS = 0.805). Within-accession variability was high, with only 16% differentiation among accessions. The results suggest that genetic diversity in *C. argentea* is relatively homogeneously distributed, indicating the likelihood of extensive outcrossing. Genebank multiplication protocols should be re-considered and the rate and impact of outcrossing measured using codominant markers. The genetic diversity of original accessions should be assessed to determine if outcrossing has occurred during or before *ex situ* storage. This might also support any decision on whether maintaining individual accessions is desirable instead of bulking.

Keywords: *Cratylia argentea*, forage legume, genetic diversity, molecular markers, outcrossing, RAPD

(Published as: Andersson *et al.* (2006b) in Plant Genetic Resources: Characterization and Utilization)

Introduction

Cratylia argentea (Desv.) O. Kuntze (syn. *C. floribunda* Benth., *Dioclea argentea* Desv.) is a drought-tolerant tropical multipurpose shrub legume with high potential particularly for dry-season supplementation and silage, due to its high nutritive value (Schultze-Kraft and Peters 1997; Argel and Lascano 1998; Lascano *et al.* 2002). The leafy shrub, native to a broad range of habitats from western Peru to the state of Ceará in Brazil, usually reaches a height of 1.5 to 3 m and remains green and productive during dry seasons up to seven months. It is well adapted to acid soils of low to medium fertility and to elevations up to 1200 m asl (Xavier *et al.* 1995; Maass 1996; Peters and Schultze-Kraft 2002).

C. argentea contains only traces of tannins and its nutritive value is higher than that of most other shrub legumes adapted to acid soils (Lascano 1996; Schultze-Kraft 1996; Shelton 2001). The species shows excellent regrowth after cutting and can be used as forage, soil cover, mulch and green manure (Argel and Lascano 1998; Lascano *et al.* 2002). It is a valuable protein source in livestock production systems, particularly during the dry season, both in cut-and-carry (fresh fodder and silage) and in grazing systems (Schultze-Kraft and Peters 1997; Argel and Lascano 1998; Jiménez *et al.* 2001; Holmann *et al.* 2002; Lascano *et al.* 2002).

Cratylia argentea can be easily propagated by seed, whereas vegetative propagation was unsuccessful (Pizarro *et al.* 1996). Collections of *C. argentea* are held by Embrapa Recursos Genéticos e Biotecnologia, Brasília, Brazil (45 accessions), Embrapa Cerrados, Planaltina, Brazil (48 accessions), and the International Centre for Tropical Agriculture (CIAT), Cali, Colombia (57 accessions) (IPGRI 2005). However, a reconciled total collection probably comprises not more than 50 different accessions.

A commercial variety (mixture of accessions CIAT 18516 and 18668) has been released in Costa Rica as cv. Veraniega (Argel *et al.* 2001) and in Colombia as cv. Veranera (Lascano *et al.* 2002). Recently, an agronomic evaluation of a collection of 38 accessions led to the identification of two new promising accessions, CIAT 18674 and CIAT 22406, with higher dry matter (DM) production, earliness and seed yield than the commercial cultivar (Chapter 2, Andersson *et al.* 2005c).

In *C. argentea*, available accessions look very similar although they were collected in a diversity of environments. A molecular marker analysis with random amplified polymorphic DNA (RAPD) was therefore conducted with a collection of 47 accessions, in order to a) assess and describe the genetic diversity within the collection, and b) within accessions, and c) identify possible genotype duplicates and taxonomic mismatches.

Materials and methods

Plant material and DNA extraction

Forty-seven *Cratylia argentea* accessions, collected mainly in the Brazilian states of Mato Grosso and Goiás, were used in this study (Table 3.1). *C. mollis* CIAT 7940 was included as reference accession for further comparison purposes. *C. mollis* is a drought tolerant shrub forage legume closely related to *C. argentea* and native to the dry *Caatinga* of north-eastern Brazil (Queiroz and Coradin 1996; Sousa and Oliveira 1996).

Accessions are bulk samples representing natural populations at their respective collection sites. Seedlings were raised in the greenhouse and transplanted to the field at the CIAT-Quilichao Experimental Station. Young leaves were harvested in the field just before reaching a fully developed size. For the determination of genetic variability among accessions, bulked samples were taken from three individual plants per accession. For the exemplary assessment of within-accession genetic variability, leaves were harvested from ten individual plants of five accessions (CIAT 18516, CIAT 18668, CIAT 18674, CIAT 22408 and CIAT 22409).

Samples were macerated in liquid nitrogen and total genomic DNA extracted from 50 mg tissue using a small-scale DNA extraction method (Qiagen DNeasy® Plant Mini Extraction Kit) with minor modifications: 600 instead of 400 µl Buffer AP1, 2 instead of 4 µl RNAse, and 150 instead of 130 µl Buffer AP2. DNA was quantified by means of a DyNA Quant™ 200 fluorometer (Hoefer Scientific Instruments, San Francisco, USA) and diluted to a final concentration of 5 ng DNA μl^{-1}.

RAPD markers

The protocol for RAPD analysis was adapted from Welsh and McClelland (1990) and Williams *et al.* (1990). The volume of the final reaction (25 µl) was composed of 1X PCR buffer (50 m*M* KCl, 10 m*M* Tris-HCl pH 8.8, 0.1% Triton x-100), 2.5 m*M* $MgCl_2$, 0.2 m*M* of each dNTPs, 0.2 µ*M* primer (Series OPD, OPG, OPI and OPJ from Operon Technologies, Alameda, CA, USA), 1 U Taq DNA polymerase (Promega, USA) and 25 ng of template DNA.

A negative control without template DNA was included in each round of reactions. Amplifications were performed in a thermocycler (PTC-100™, MJ Research Inc.) with an initial denaturing step of 5 min at 94 °C followed by 40 cycles of 30 s at 94 °C, 30 s at 38 °C and 1 min at 72 °C and a final extension step of 5 min at 72 °C. The PCR products were run on a 1.4% agarose gel at 20 V cm^{-1} during 45 min. The amplified DNA fragments were visualised by ethidium bromide staining (0.5 µg ml^{-1} bromide in gel and buffer) under UV-light, and photographed with a Kodak digital camera DC 120 (Software Kodak Digital Science™ 1997).

Table 3.1 Origin of the *Cratylia argentea* accessions and *C. mollis* used in the present study

Accession (CIAT No.)	Accession (BRA[a] No.)	State	Latitude	Longitude	Altitude (m asl)	Reference[b]
18516	000167	Goiás	13° 22' S	46° 25' W	800	2
18667	000027	Mato Grosso	15° 43' S	55° 43' W	455	1
18668	000035	Mato Grosso	15° 22' S	56° 13' W	175	1
18671	000060	Mato Grosso	14° 46' S	57° 05' W	230	1
18672	000086	Pará	03° 45' S	55° 14' W	140	2
18674	000116	Mato Grosso	14° 38' S	52° 22' W	320	3
18675	000124	Mato Grosso	14° 54' S	52° 17' W	380	3
18676	000132	Goiás	16° 21' S	51° 20' W	450	3
18957	000175	Tocantins	06° 30' S	48° 37' W	350	2
22373	000591	Goiás	14° 05' S	46° 23' W	780	3
22374	000612	Goiás	13° 16' S	46° 25' W	660	3
22375	000639	Goiás	13° 01' S	46° 36' W	620	3
22376	000655	Goiás	14° 23' S	49° 09' W	550	3
22377	000663	Goiás	13° 54' S	49° 03' W	510	3
22378	000671	Goiás	13° 37' S	49° 02' W	390	3
22379	000680	Goiás	13° 21' S	47° 07' W	380	3
22380	000698	Goiás	13° 14' S	49° 28' W	340	3
22381	000701	Goiás	13° 14' S	50° 40' W	360	3
22382	000710	Goiás	13° 17' S	50° 12' W	330	2
22383	000728	Goiás	13° 51' S	50° 20' W	300	2
22384	000736	Mato Grosso	14° 14' S	52° 10' W	360	3
22386	000752	Mato Grosso	14° 34' S	52° 21' W	320	3
22387	000761	Mato Grosso	15° 50' S	52° 25' W	370	3
22388	000787	Mato Grosso	16° 23' S	54° 01' W	330	3
22389	000795	Mato Grosso	16° 26' S	54° 19' W	400	3
22390	000809	Mato Grosso	16° 01' S	54° 55' W	300	3
22391	000817	Mato Grosso	15° 49' S	55° 30' W	400	3
22392	000825	Mato Grosso	15° 58' S	55° 00' W	240	3
22393	000833	Mato Grosso	15° 42' S	56° 42' W	210	3
22394	000868	Mato Grosso	15° 52' S	57° 49' W	200	3
22395	000892	Goiás	16° 25' S	51° 35' W	460	3
22396	000906	Goiás	16° 32' S	51° 03' W	510	3
22397	'Yapacaní'	Bolivia, Santa Cruz	17° 24' S	63° 56' W	125	2
22399	000604	Goiás	14° 30' S	46° 24' W	650	3
22400	000621	Goiás	13° 10' S	46° 40' W	660	3
22401	000841	Mato Grosso	15° 51' S	56° 49' W	360	3
22402	000876	Mato Grosso	16° 30' S	54° 36' W	270	3
22403	000884	Mato Grosso	16° 34' S	51° 45' W	600	3
22404	000191	Goiás	13° 01' S	56° 37' W	650	3
22405	000213	Goiás	13° 15' S	46° 28' W	700	3
22406	000221	Goiás	14° 15' S	46° 30' W	780	3
22407	000514	Goiás	14° 54' S	46° 56' W	500	3
22408	000540	Mato Grosso	14° 06' S	46° 25' W	810	3
22409	000566	Goiás	13° 27' S	46° 22' W	540	3
22410	000574	Goiás	13° 17' S	46° 25' W	660	3
22411	000647	Goiás	15° 12' S	46° 47' W	550	3
22412	000779	Mato Grosso	15° 42' S	52° 43' W	400	3
7940	*C. mollis*	n.a.	n.a.	n.a.	n.a.	2

[a] BRA, Embrapa Recursos Genéticos e Biotecnologia, Brasília, Brazil.

[b] 1, Queiroz and Coradin (1996); 2, Barco *et al.* (2002); 3, G.P. da Silva (Embrapa Recursos Genéticos e Biotecnologia), pers. comm. April 2005); n.a., not available

Table 3.2 Oligonucleotide primers employed in RAPD analysis, their sequence, number of polymorphic (P) and monomorphic (M) bands obtained, and percentage of polymorphic bands (% PB)

Primer code	Sequence (5' to 3')	Number of bands (including *C. mollis*)		(only *C. argentea*)	
		P	M	P	M
OPD 15	CATCCGTGCT	9	1	7	1
OPG 12	CAGCTCACGA	16	1	11	1
OPI 07	CAGCGACAAG	16	1	12	2
OPJ 06	TCGTTCCGCA	13	0	13	0
OPJ 07	CCTCTCGACA	8	0	8	0
OPJ 12	GTCCCGTGGT	6	1	5	1
Sum		*68*	*4*	*56*	*5*
Total		*72*		*61*	
PB (%)		*94.4*		*91.8*	

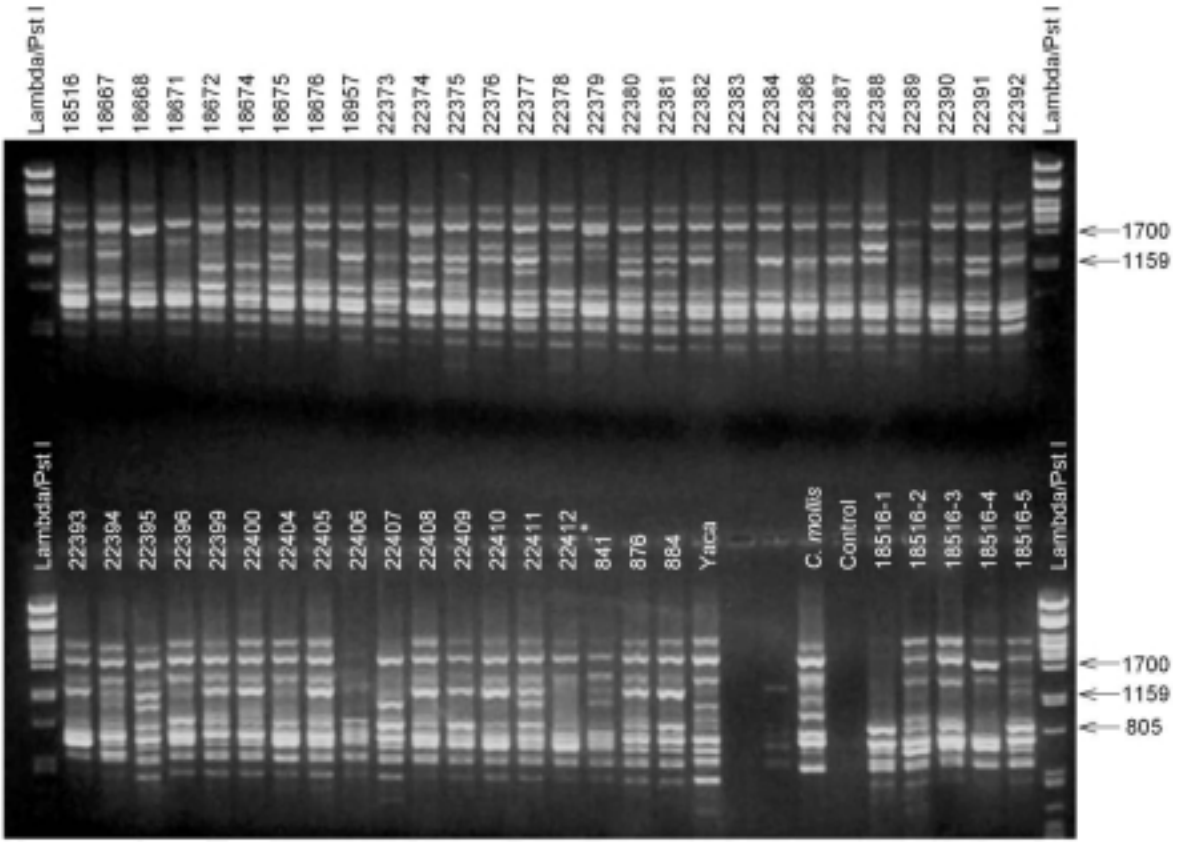

Figure 3.1 RAPD profile obtained using the primer OPD15 for the 47 *Cratylia argentea* genotypes, *C. mollis*, one DNA-free control, and five samples of different individuals of accession CIAT 18516 for the assessment of within-genotype variability. Size markers (λ-DNA/PstI, Invitrogen, USA) for assessing base pair lengths are shown in the first and last lane of each row

Statistical analysis

Amplified DNA fragments were manually scored as present (1) or absent (0) for each primer, assuming that each band position corresponds to a genetic locus with two alleles, distinguishing presence and absence of the band, respectively (Lynch and Milligan 1994). Data were converted into a similarity matrix using the Nei and Li similarity coefficient based on the proportion of shared alleles (Nei and Li 1979), adapted from Dice (1945). The Nei and Li similarity coefficient calculates the genetic similarity (*GS*) between two samples, *i* and *j*, with the formula $GS(i,j) = 2N_{ij} / (2N_{ij} + N_i + N_j)$, where N_{ij} is the number of bands present in both *i* and *j*, N_i is the number of bands present in *i* and absent in *j*, and N_j is the number of bands present in *j* but absent in *i*. Heterogeneity was determined using Nei's *H* and G_{ST} estimators (Nei 1973).

Then, a similarity tree was produced by clustering the similarity matrix based on the Average Linkage UPGMA (unweighted pair group method with arithmetic averages) algorithm of Sneath and Sokal (1973). In addition, multiple correspondence analysis (MCA) was performed on the original matrix in order to visualize the dispersion of individuals in relation to the first three principal axes of variation. Cluster analysis was performed using the NTSYS package version 2.1 (Rohlf 2000); for all other statistical analyses SAS version 8.2 (SAS Institute Inc. 1999) was used.

Results

RAPD profiles

Of the 47 oligonucleotide decamer primers initially screened with three *C. argentea* accessions, 17 gave smeared or faint bands and seven gave no amplification products. Fifteen primers identified high levels of polymorphisms and were repeated to test for reproducibility. Of these, six primers detected distinct, clearly resolved and consistently reproducible amplification products and were, therefore, selected for the amplification of RAPD sequences. They generated a total of 72 scorable fragments across the 47 *Cratylia argentea* genotypes and the *C. mollis* reference accession (Table 3.2). On average, 11 scorable fragments were obtained per primer, ranging from six (primer OPJ 12) to 16 (primers OPG 12 and OPI 07). The band size ranged from 320 to 2900 bp. A RAPD profile generated by primer OPD 15 is shown in Figure 3.1.

Of the 72 fragments detected across all genotypes analysed, 68 (94%) were polymorphic. Among *C. argentea* accessions, 61 fragments were detected, 56 (92%) being polymorphic. Eleven markers generated by primers OPD 15, OPG 12, OPI 07 and OPJ 12 were unique to *C. mollis*, and five markers (all generated by primer OPI 07) were unique to *C. argentea* accessions. Of these, three were unique to the only prostrate *C. argentea* accession CIAT 22397 'Yapacaní', and the other two were found in CIAT 22399 and CIAT 22403,

respectively. In addition, three markers (generated by primers OPJ 06 and OPJ 12) were common to *C. mollis* and 'Yapacaní', but were absent in the other *C. argentea* genotypes. Regarding the geographic distribution of the fragments, no fragments unique to the two northern most accessions, i.e. those that were geographically most distant from the rest of the collection, CIAT 18672 (03° 45' S) and CIAT 18957 (06° 30' S) were detected.

Cluster and multiple correspondence analysis

Cluster analysis based on genetic similarity separates the 47 *Cratylia argentea* accessions and the *C. mollis* reference into seven groups (Figure 3.2). First, *C. mollis* is separated, followed by the only prostrate accession 'Yapacaní' (CIAT 22397). Subsequently, two groups containing only one accession each are split: CIAT 18668 and CIAT 22389. Next, one single accession CIAT 22408, and a group comprising accessions CIAT 18674, CIAT 22384, CIAT 22386 and CIAT 22401 are separated. The remaining 39 accessions (81%) fall into one main cluster (Figure 3.2).

Multiple correspondence analysis broadly confirms the pattern of cluster analysis, differing only slightly in some details (Figure 3.3). The first and second dimensions which account for 27% and 14% of the total variation, respectively, clearly separate the *C. mollis* reference and the prostrate *C. argentea* accession 'Yapacaní' from the remaining genotypes. The multiple correspondence coordinates of each of the three groups (*C. mollis*, 'Yapacaní' and the remaining *C. argentea* genotypes) form the extreme points of an almost equally-sided triangle (Figure 3.3A), indicating that the prostrate *C. argentea* accession 'Yapacaní' is genetically almost as distant from all other *C. argentea* genotypes as is *C. mollis*. The third dimension helps understand the structure of the remaining 46 *C. argentea* accessions, discriminating them into two main and three smaller groups (Figure 3.3B). Accession CIAT 18668 is grouped together with CIAT 22389 and CIAT 22403, and CIAT 22386 with CIAT 22387. CIAT 18674 makes up a group of its own. The remaining 40 accessions (83%) fall into two main clusters, containing 28 and 12 accessions, respectively. The 28 accessions composing Group 1 are widely distributed throughout the states of Goiás and Mato Grosso, whereas seven out of the 12 accessions comprising Group 2 were collected in the east of the state Goiás with a maximum distance of 60 km among accessions.

Genetic diversity and genetic similarity among and within groups

Total genetic diversity in the sample studied here was low (H_T = 0.169), with only 30% of differentiation among groups (G_{ST} = 0.302) (Table 3.3). The mean genetic similarity was high (*GS* = 0.776) and ranged from 0.388 to 0.814 among groups (Table 3.4) and from 0.316 to 0.947 among accessions (data not shown).

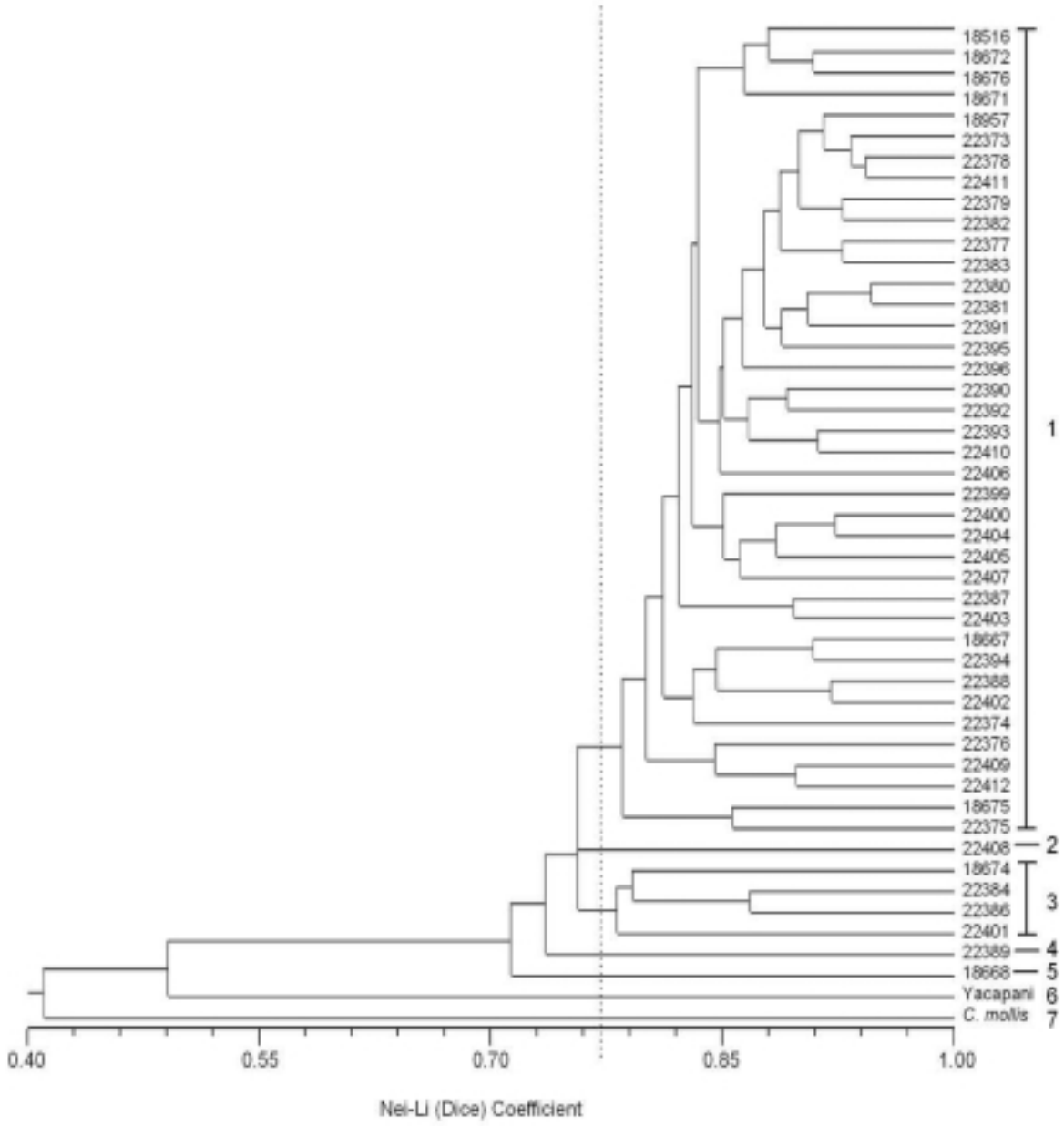

Figure 3.2 Grouping of 47 *Cratylia argentea* and a *C. mollis* reference accession using UPGMA. Genetic distances are according to Nei and Li based on RAPD markers

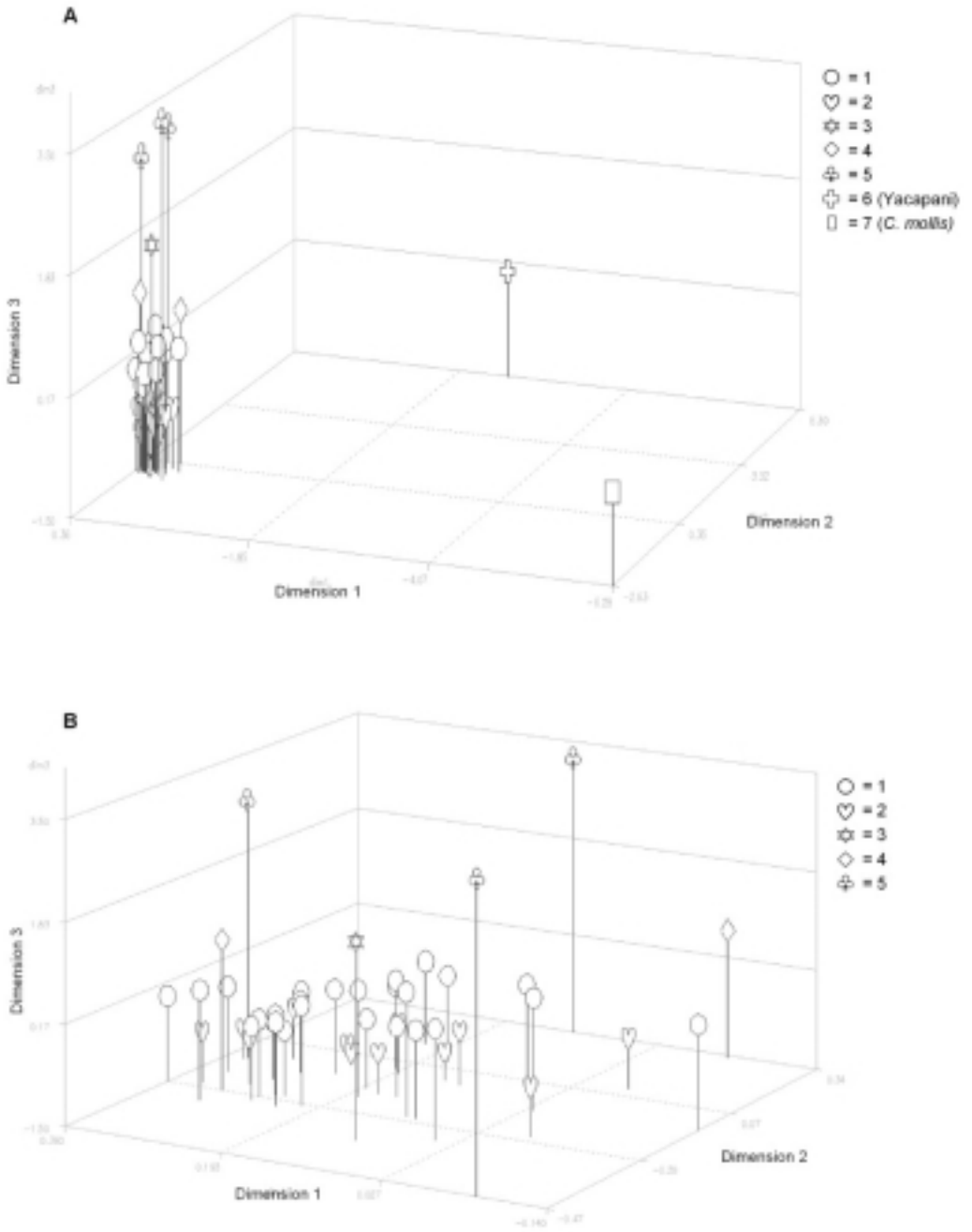

Figure 3.3 Three-dimensional representation of seven groups derived from multiple correspondence analysis of RAPD markers among 47 *Cratylia argentea* accessions and one *C. mollis* accession (A). (B) shows a higher resolution of Groups 1 to 5 (without 'Yapacaní' and *C. mollis*)

Table 3.3 Nei estimates of genetic diversity (heterogeneity) among groups

Group	*n*	$H_{i(1-7)}$	$H_{i(1-5)}$
1	28	0.128	0.128
2	12	0.126	0.126
3	1	0.0	0.0
4	2	0.101	0.101
5	3	0.120	0.120
'Yapacaní'	1	0.0	-
C. mollis	1	0.0	-
H_S		*0.118*	*0.123*
H_T		*0.169*	*0.145*
G_{ST}		*0.302*	*0.152*

n, number of accessions; H_T, total genetic diversity; H_i, genetic diversity within each group; H_S, average genetic diversity within groups; G_{ST}, coefficient of genetic differentiation (proportion of total genetic diversity found among groups)

Table 3.4 Average genetic similarity (*GS*) values between (above diagonal) and within (diagonal) groups of *Cratylia argentea* germplasm, the prostrate accession 'Yapacaní' and the *C. mollis* reference accession, based on all pair wise similarities between genotypes according to molecular marker information (RAPD)

		Cratylia argentea							
Group	N	1	2	3	4	5	*Total*	'Yapacaní'	*C. mollis*
1	28	0.825	0.814	0.769	0.774	0.759		0.487	0.413
2	12		0.839	0.720	0.764	0.721		0.515	0.404
3	1			1.000	0.757	0.754		0.426	0.433
4	2				0.717	0.748		0.479	0.400
5	3					0.757		0.457	0.388
C. argentea	*46*						*0.805*		
'Yapacaní'	1							1.000	0.444
C. mollis	1								1.000
Total	*48*								*0.776*

Table 3.5 Genetic similarity among (above diagonal) and within (diagonal) five *Cratylia argentea* accessions, based on all pair wise similarities between individuals according to molecular marker information (RAPD)

Accession (CIAT No.)	N	18516	18668	18674	22408	22409	Total
18516	10	0.730	0.704	0.724	0.669	0.735	
18668	10		0.763	0.727	0.667	0.719	
18674	10			0.782	0.692	0.753	
22408	10				0.737	0.710	
22409	10					0.800	
Total	*50*						*0.720*

When excluding the two genetically most distant accessions, 'Yapacaní' and *C. mollis* (Groups 6 and 7, respectively), total diversity decreased to $H_T = 0.145$, and the coefficient of gene differentiation was reduced by half to 15.2% (Table 3.3). Mean *GS* increased to 0.805, ranging from 0.720 to 0.814 among groups (Table 3.4) and from 0.604 to 0.947 among accessions (data not shown). The genetic similarity between groups (Table 3.4, above diagonal) was as high as or even higher than within groups (Table 3.4, diagonal).

Geographic origin (i.e. distance) had only limited influence on the composition of the groups. In several instances, genetically similar accessions were collected from geographically very distant areas (e.g. CIAT 22373 and CIAT 22409), whereas geographically close accessions often appeared to be genetically very distant in both cluster and MCA (e.g. CIAT 18675 and CIAT 22408). Also, it was not possible to distinguish the two northern most accessions CIAT 18672 and CIAT 18957 which clustered together tightly with 37 other accessions distributed widely throughout Central Brazil.

Gene diversity according to altitude

As no clear structure of genetic diversity was detected among groups, genetic diversity among accessions collected at sites with similar elevations was analyzed. Therefore, two groups were defined, one containing those accessions collected at elevations from 0 to 500 m asl ($n = 18$), and the other from 501 to 810 m asl ($n = 22$). However, genetic diversity of these two groups was not significantly different, with less than 5% of the total genetic diversity accounting for by divergence in elevation ($H_T = 0.145$, $H_S = 0.138$, $G_{ST} = 0.048$).

Genetic diversity and genetic similarity within accessions

To compare mean genetic diversity within and among *C. argentea* accessions ten individual plants of each of five accessions were chosen. Total genetic diversity H_T among these accessions was 0.180, while mean genetic diversity within accessions (H_S) was 0.152, representing an 84% of total genetic variation. Mean genetic similarity *GS* was 0.720, ranging from 0.730 to 0.800 within the five accessions studied (Table 3.5).

Possible duplicates in the collection

If considering accessions as genetically identical when their genetic similarity *GS* was equal to or greater than 0.95, then all genotypes were uniquely identified, indicating that the collection does not contain genetic duplicates. However, four pairs of possible duplicate candidates (CIAT 22380/22381, CIAT 22381/22411, CIAT 22378/22411 and CIAT 22373/22378) were identified, since their pairwise genetic similarity was very close to this value ($GS > 0.94$).

Discussion and conclusions

In this first study of genetic diversity in *Cratylia* with a molecular technology, the level of polymorphism detected with six decamer RAPD primers was very high (>90%) and allowed the distinction of all accessions analysed. The high discrimination power indicates that the RAPD technology provides an effective tool for germplasm analysis in *Cratylia*.

Genetic structure within the collection

Overall genetic diversity in the collection was low, with high genetic similarity among accessions. Cluster and multiple correspondence analysis showed the clear separation of *C. mollis* and the prostrate accession 'Yapacaní'. However, there was no clear pattern with respect to the remaining 46 *C. argentea* accessions which were divided into one main and four smaller groups. The low genetic differentiation and high genetic similarity among groups show that the low genetic diversity is fairly homogeneously distributed in the collection, without any particular pattern. This was also confirmed by the fact that neither geographic origin (i.e. distance) nor altitude had any significant influence on the composition of these groups. In addition, the genetic variability within accessions was high, with genetic similarities within accessions being only slightly greater than mean similarity among accessions. These findings suggest the existence of a single, widely shared genepool with limited genetic separation among accessions, and indicate the likelihood of extensive outcrossing. These results agree with prior studies of tropical tree population structure. Tropical tree species appear to have large genepools, with high diversity and little genetic structure, due to extensive outcrossing and geneflow occurring over distances of several kilometres (Chase *et al.* 1995; Schierenbeck *et al.* 1997).

Mating system

Among various factors that determine the genetic composition of plant populations, mating system is the most influential one (Hamrick and Godt 1990). High polymorphism levels of the magnitude observed in the present study for *C. argentea* (92%) are reported for outcrossing species, whereas predominantly selfing and/or clonal species generally show much higher proportions (45-80%) of monomorphic loci (Hamrick and Godt 1990; Bussell 1999; Zhivotovsky 1999; Forapani *et al.* 2001). Similarly, the relatively low differentiation among groups and the high genetic diversity within groups, are characteristic for predominantly outcrossing species, whereas for inbreeding species this relationship tends to be inverted (Hamrick and Loveless 1989; Nybom 2004).

Generally, tropical legumes have been considered as predominantly self-pollinated, but there is increasing evidence of cross-pollination in many species, suggesting that some outcrossing may occur in most legume species (Hacker and Hanson 1999). Very little is known about the genetics of *C. argentea*, and particularly about its mating system. The chromosome number of

the species is $2n = 22$ (Queiroz 1991), but it is unclear whether the reproduction system of *C. argentea* is allogamous or autogamous. The only information available is a preliminary study by Queiroz *et al.* (1997), who reported the occurrence of outcrossing and suggested a mixed mating system. The findings presented here provide further evidence for this hypothesis.

Furthermore, field observations suggest that tripping is involved in fertilization (Chapter 2). Pollinators such as the exotic honeybee *Apis mellifera* L., and other bees and bumblebees belonging to the genera *Bombus*, *Centris* and *Xylocopa* (Hymenoptera: Apoidea) have been observed to visit *C. argentea* flowers loaded with pollen (Queiroz 1996; Sobrinho and Nunes 1996; Xavier and Carvalho 1996). Some of these pollinators have been reported to be capable of flight distances of several kilometres thus effecting pollen dispersal between plants at great distances, e.g. the honeybee *A. mellifera* (Levin and Glowska-Konopacka 1963; Winston 1987) and the carpenter bee *Xylocopa fimbriata* Fabr. (Janzen 1971; Simons and Dunsdon 1992).

At CIAT, *C. argentea* accessions are multiplied in plots that are at least 300 m distant and hence, isolated from each other to a certain extent (A. Ciprián, CIAT Genetic Resources Unit, pers. communication 2005). It is thus possible that cross-pollination among accessions occurred both during multiplication in the field and within original populations in the wild.

It is therefore suggested that precautions are taken by genebanks during the regeneration of *C. argentea* accessions to avoid cross-pollination, and the re-consideration of multiplication protocols in order to maintain the genetic integrity of accessions. More detailed studies (e.g. with codominant molecular markers) are required in order to identify the prevailing reproductive strategy of *C. argentea* and to assess the rate and impact of outcrossing in this species. Furthermore, the genetic diversity measured within accessions in this study should be compared to the genetic diversity within the respective "original" accessions, in order to determine if outcrossing occurred during or before *ex situ* storage. This could be done by analysing seeds either from the original collection, or – in case no original seeds were available – re-collected from the original populations in the wild. Based on the information about the extent of genetic diversity within original accessions it should be decided whether the maintenance of individual accessions is desirable, or whether they should be pooled.

Possible duplicates and taxonomic mismatches in the collection

Although genetically all accessions were significantly different ($P < 0.05$) from each other, a group of five possible duplicate accessions (CIAT 22373, CIAT 22378, CIAT 22380, CIAT 22381, CIAT 22411) was identified which shared more than 94% of their banding pattern, differing in three bands only. Re-analysing these accessions with an additional primer

would likely lead to a clearer molecular differentiation between pairs. On the other hand, it is justifiable to consider them as genetic duplicates because an experimental error (e.g. amplification error, slightly lower or faster band movement of one of the accessions in the gel) in only one of these bands would increase genetic similarity to more than 95%. For the identification of duplicates it is important to consider not only one type of data (e.g. genetic), but rather verify duplication on the basis of additional information available, such as origin, morphological and agronomic data (Hintum *et al.* 1996). The five accessions were indeed similar morphologically as well as agronomically and were grouped into the same low-quality complex in an agronomic evaluation (Chapter 2). Thus, bulking them into a single accession is justified.

Ordination was particularly useful in visualising that the prostrate accession 'Yapacaní' was genetically almost as distinct from all other C. argentea accessions as was C. mollis. Re-classification of the 'Yapacaní' accession is required in order to determine its taxonomic status. The fact that its original-collection site (the southern-most and western-most of the whole collection) is quite distant from that of all other accessions is a further evidence for Yapacaní belonging to a different taxon.

With respect to accessions CIAT 18516 and CIAT 18668 forming the commercial variety, it was striking that CIAT 18668 was genetically very different from CIAT 18516 as well as from most other *C. argentea* accessions (Figure 3.2). In agronomic evaluations, however, these two accessions were not only very similar regarding morphological characteristics and forage quality (Chapter 2; Schultze-Kraft 1996). They also showed consistently high DM production in different environments (Maass 1996) and were, therefore, chosen for release as commercial cultivar (Lascano et al. 2002). This may indicate that the genetic diversity detected in this study might be mainly due to polymorphisms of neutral markers and thus might not have any agronomic implications.

Chapter 4

Morphological and phenological characterization of the tropical shrub legume *Flemingia macrophylla* (Fabaceae, Papilionoideae)

Abstract

Flemingia macrophylla (Willd.) Merrill is a drought-tolerant, tropical multipurpose shrub legume especially suited to low-input smallholder production systems, and is used as dry season forage supplement, live soil cover, mulch and living barrier, among others. The diversity of the world collection of 70 accessions classified as *F. macrophylla* was assessed using 14 morphological and phenological traits. One erect and three semi-erect morphotypes were identified based on differences in plant height, flower and seed colour, inflorescence and peduncle length, and stem pubescence: Morphotype M1 (n = 19), erect accessions with stalked inflorescences and black seeds; M2 (n = 23), semi-erect accessions with dense, congested sessile racemes, dark pink flowers, glabrous or slightly pubescent stems and mottled brown seeds; M3 (n = 5), semi-erect accessions with lax sessile racemes, less than 20 pink or white flowers per raceme and mottled brown seeds; and M4 (n = 20), semi-erect accessions with densely congested, sessile racemes, light pink flowers, strongly pubescent stems and mottled brown seeds. Flowering is probably induced by reduction of day length and water stress. Flowering and seeding behaviour of all accessions was very similar, and no biological (temporal) barriers to cross-pollination were detected among the morphotypes. Further morphological studies on inflorescence characteristics of herbarium specimens and molecular marker studies are warranted to clarify the taxonomic status (subspecies or varieties) of the four morphotypes identified.

Keywords: flowering induction, morphotypes, *Flemingia macrophylla*, tropical legumes, morphological characterization, phenology, taxonomy

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Introduction

The genus *Flemingia* Roxb. ex W. T. Aiton comprises erect or prostrate shrubs and herbs native to the tropics and subtropics (Thuân 1979; Verdcourt 1979). There are about 40 species, but the taxonomic status of many of them is not clear (Maesen 2003; ILDIS 2005) and the genus is currently under revision (L.J.G. van der Maesen, personal communication 2005). The agronomically most important species is *Flemingia macrophylla* (Willd.) Merrill (syn. *F. congesta*, *Moghania macrophylla*), a perennial, drought-tolerant, multipurpose shrub legume growing up to 4 m high which is especially suited to low-input smallholder production systems in the sub-humid and humid tropics. The species enhances soil fertility and is used for a number of purposes such as dry season forage supplement, live soil cover or mulch, erosion barrier hedge, shade-providing shrub in young coffee and cocoa plantations, and as firewood (Andersson *et al.* 2002; Maesen 2003). Considerable morphological variation among *F. macrophylla* accessions and even different growth habits can be observed in the field, but previous research concentrated on only a few accessions. Since a larger germplasm collection is available, a characterization based on morphological and phenological traits is justified. Different growth habits or morphotypes have so far been reported only for a few tropical shrub or tree legumes, e.g. *Leucaena leucocephala* (Lam.) de Wit (Dommergues *et al.* 1999), *Calliandra calothyrsus* Meisn. (Chamberlain 1998; Macqueen 2001) and *Desmodium velutinum* (Willd.) DC. (N. Rivas, personal communication 2005).

The objective of the present study was to assess the diversity in the world collection classified in germplasm databases as *Flemingia macrophylla* (Willd.) Merrill, in terms of morphological and phenological parameters, in order to a) facilitate the selection of accessions for further agronomic evaluation, and b) provide information relative to live plants for eventual taxonomic considerations, as a contribution to the current revision of the genus.

Materials and methods

Evaluation site

The collection comprised 70 accessions assembled mainly from Southeast Asia and held at the CIAT Genetic Resources Unit (Table 4.1). The trial was established at the CIAT-Quilichao Experimental Station in the Cauca department, Colombia (03°06' N, 76°31' W; 990 m asl; average annual temperature 23 °C).

Annual rainfall is distributed in a bimodal pattern with two generally well-pronounced rainy seasons from March to June and from September to December, with an average of 1800 mm per year. During the establishment period (May - December 2001), rainfall was 816 mm, and 1265 mm during the subsequent experiment year. The soil characteristics of the experimental plot (Ultisol) were pH = 5.3, 76% Al saturation, medium P (6 ppm Bray II) and high organic matter content (7.4%).

Table 4.1 Geographic origin of the 70 *Flemingia macrophylla* accessions used in the study

Origin	Accession (CIAT No.)
Southeast Asia	
China (10)	
Hainan Island	18048, 20972, 20973, 20975, 20976, 20977, 20978, 20979, 20980, 20982
Vietnam (9)	
North Central Coast	21982
South Central Coast	21990, 21991, 21992, 21993, 22285
Central Highlands	21995, 21996, 22327
Thailand (14)	
Northern Thailand	21083, 21087, 21090
Northeastern Thailand	17400, 22082, 22087, 22090
Central Thailand	21079
Eastern Thailand	18438, 18440
Southern Thailand	17403, 17404, 17405, 17407
Peninsular Malaysia (4)	17409, 17411, 17412, 17413
Indonesia (19)	
Aceh, Sumatra	20616, 20617, 20618, 20621
North Sumatra	20622, 20623, 20624, 20626, 20631
West Sumatra	18437, 19797, 19798, 19824, 20625
Jambi and South Sumatra	19799, 19800, 19801
Java	21529
Rote	20065
Papua New Guinea (3)	19453, 19454, 19457
India (1)	CPI-104890
Africa	
Cameroon (1)	21580
Ghana (1)	21248
Central and South America	
Colombia (3)	20744, 21241, J-001
Costa Rica (1)	I-15146
Pacific	
Hawaii/USA (1)	21519
Without origin information (3)	801, 7184, 21249

Seeds were sown in Jiffy pots (February 2001), inoculated with *Bradyrhizobium* strain CIAT 4099, and seedlings transplanted to the field after ten weeks, in May 2001. Four plants per accession were established in single-row plots, with a distance of 1 m between plants where individual plants stood as replicates. Four weeks after transplanting, fertilizer (40:50:20 kg ha^{-1} of P:K:Mg, Mo 500 g ha^{-1}) was applied to single plants. Eight weeks after a standardization cut, plants were cut at a height of 15 cm for low-growing accessions and 30 cm for all others and then allowed to flower and set seed.

Morphological and phenological evaluation

The collection was characterized using a set of 14 attributes related to morphology, phenology and seed production (Table 4.2). Special attention was paid to taxonomically relevant traits used in floras for the distinction among different *Flemingia* species, such as congested or lax inflorescences, length of superior calyx lobes and inflorescence length:petiole length ratio (Merrill 1910; Thuân 1979; Verdcourt 1979). The values presented here are the means of measurements or observations on three plants (= replications) per accession.

Time to flowering was recorded after sowing, when 50% of the buds were flowering. Flower colour (D = dark pink, L = light pink, W = white) and the number of flowers per inflorescence (1 = < 20, 2 = ≥ 20) were recorded. Inflorescence and peduncle lengths (mm) were measured by selecting ten average-sized, fully developed inflorescences per plant, and the length of the superior calyx lobes was recorded in relation to the length of the corolla (1 ≅ corolla, 2 = << corolla).

Plant height and diameter (cm) were measured after 14 months, as well as the total leaf area of the three leaflets (cm^2) and the petiole length (mm) by selecting ten average-sized, fully developed leaves from the middle part of each of the three plants, and stem and petiole pubescence was recorded (G = glabrous to slightly pubescent, P = strongly pubescent). After cutting, total cumulative seed yield (g) per plant (obtained by handpicking of all ripe pods twice a week during four months) and seed colour (B = shiny black, M = mottled brown) were recorded. The weight of randomly sampled 100 seeds (three replications) was measured and subsequently 1000-seed weight (g) calculated. Twenty accessions did not produce sufficient seed to calculate 1000-seed weight, and seeds stored in the CIAT Genetic Resources Unit were used instead. The trial was concluded six months after the second cut, although not all accessions had flowered by that time.

Statistical analysis

Data were subjected to principal component analysis (PCA). Correlations were calculated and when the correlation coefficient (*r*) between two variables was greater than 0.7, then only one of these was included in the subsequent cluster analysis (Ward's method, SAS Institute Inc. 1999). Accession CIAT 21087 was the only accession which flowered neither after sowing nor after cutting, and was excluded from cluster analysis.

Results

The majority of the accessions (61 accessions) were well adapted to the experimental site, except CIAT 20065 and CIAT 22087 which did not survive. Only one plant per accession established for accessions CIAT 18437, CIAT 18438, CIAT 19454, CIAT 19797,

CIAT 21249 and CIAT 21996, probably due to low seed quality. After cutting, accessions CIAT 18437, CIAT 21087 and CIAT 21995 were lost during the dry season.

Great variability in terms of growth, flowering and seed characteristics was detected among accessions (Table 4.3 and Table 4.4). Plant height after 14 months varied from 40 to 287 cm (average 148 cm), and plant diameter from 90 to 280 cm. Inflorescences were either sessile (i.e. without peduncles) or stalked, with the peduncle length varying from 20 to 50 mm. Inflorescence length ranged from 18 to 95 mm (average 50 mm).

Table 4.2 Morphological and phenological attributes recorded in the *Flemingia macrophylla* collection

Attribute	Unit of assessment	Time of assessment
Time to flowering	No. of days after sowing	at 50% of buds flowering
Inflorescence length	mm	at 50% of buds flowering
Peduncle length	mm	
Flower colour	D=dark pink, L=light pink, W=white	
Length of calyx lobes	1 ≅ corolla, 2 = << corolla	
No. of flowers per inflorescence	1 = < 20, 2 = ≥ 20.	
Leaf area	cm^2	14 months after sowing (before standardization cut)
Petiole length	mm	
Stem and petiole pubescence	G = glabrous to slightly pubescent, P = strongly pubescent	
Plant height	cm	
Plant diameter	cm	
Seed yield	g $plant^{-1}$	after cutting
Seed colour	B = shiny black, M = mottled brown	
Seed weight	g 1000-$seeds^{-1}$	

Table 4.3 Growth, flowering and seed characteristics of *Flemingia macrophylla* accessions at the CIAT- Quilichao Experimental Station, Colombia

	n	Range	Mean	SEM*
Plant height (cm, after 14 months)	68	40 - 287	148	9.2
Plant diameter (cm, after 14 months)	68	90 - 280	178	5.0
Ratio height:diameter	68	0.3 - 1.6	0.8	0.04
Inflorescence length (mm)	67	18 - 95	50	2.2
Peduncle length (mm)	67	0 - 50	8	1.7
Leaf area (cm^2)	68	31 - 216	97	4.1
Petiole length (mm)	68	26 - 80	49	1.4
Ratio inflorescence:petiole length	67	0.4 - 1.9	1.1	0.05
Days to flowering (after sowing)	68	180 - 388	259	4.8
Average seed weight (g 1000-$seeds^{-1}$)	70	8 - 24	17	0.3
Seed yield (g $plant^{-1}$)	50	0 - 140	40	4.9

* SEM, standard error of the mean

The average time to flowering after sowing was 259 days, and 290 days to pod setting. After cutting, flowering occurred more than four months earlier (after 126 days on average) than after sowing. The earliest accession after sowing was CIAT 20631 (180 days) followed by CIAT 17405, CIAT 20972, CIAT 21995 and CIAT 22285 (the latter were all 199 days). Flower colour was either dark or light pink, and accession CIAT 22082 had white flowers. The seeds were either shiny black or mottled brown, and seed weight ranged from 8 to 24 g 1000^{-1} seeds. Average seed production during four months varied from 0 to 140 g $plant^{-1}$ (average 40 g $plant^{-1}$).

The cluster dendrogram was truncated at the 7-group level, explaining 72% of total variation (Figure 4.1). Cluster analysis divides the 67 accessions into four principal groups at the 52%-level, which from now on will be referred to as "morphotypes" (M), with one group containing erect accessions (M1) and three groups with different types of semi-erect accessions (M2, M3 and M4). The attributes that contributed most strongly to the separation of these morphotypes were plant height, inflorescence and peduncle length, flower and seed colour, and stem pubescence (Table 4.5). The primary splitting separates the first group of 19 accessions (Group M1, see Figure 4.1) with erect growth habit, long and stalked inflorescences with numerous light pink flowers and black seeds, the inflorescence length exceeding the length of the leaf petiole. All remaining accessions are semi-erect, with unstalked (sessile) inflorescences and mottled brown seeds. They are divided into three different groups.

Group M2 consists of 23 accessions with shorter and densely congested, sessile inflorescences composed of numerous dark pink flowers. The inflorescence usually equals the leaf petiole in length, and the stem and petiole are always strongly pubescent. Group M3 is composed of five semi-erect accessions with sessile, very lax racemes having few (usually < 20) dark pink flowers (white in one case), the racemes usually being shorter than the leaf petiole. The stem and petioles are glabrous or slightly pubescent. These accessions flowered after 344 days, which was the latest at the experimental site, nearly three months later than the average. The flowers were caducous and abscised before fertilisation occurred, thus resulting in very low seed production (4 g $plant^{-1}$, compared to an average of 40 g $plant^{-1}$ for the whole collection).

The 20 semi-erect accessions of Group M4 resemble in their general growth habit very much those of Group M2. The main differences are: smaller plant height, light pink flowers (Group M2 has dark pink flowers), smaller leaves (average leaf area of Group M4 is 73 cm^2, as compared to 106 cm^2 in Group M2), and strongly pubescent stems and petioles (Group M4 has glabrous or slightly pubescent stems and petioles). Other taxonomically important differences among these four major groups are summarized in Table 4.5.

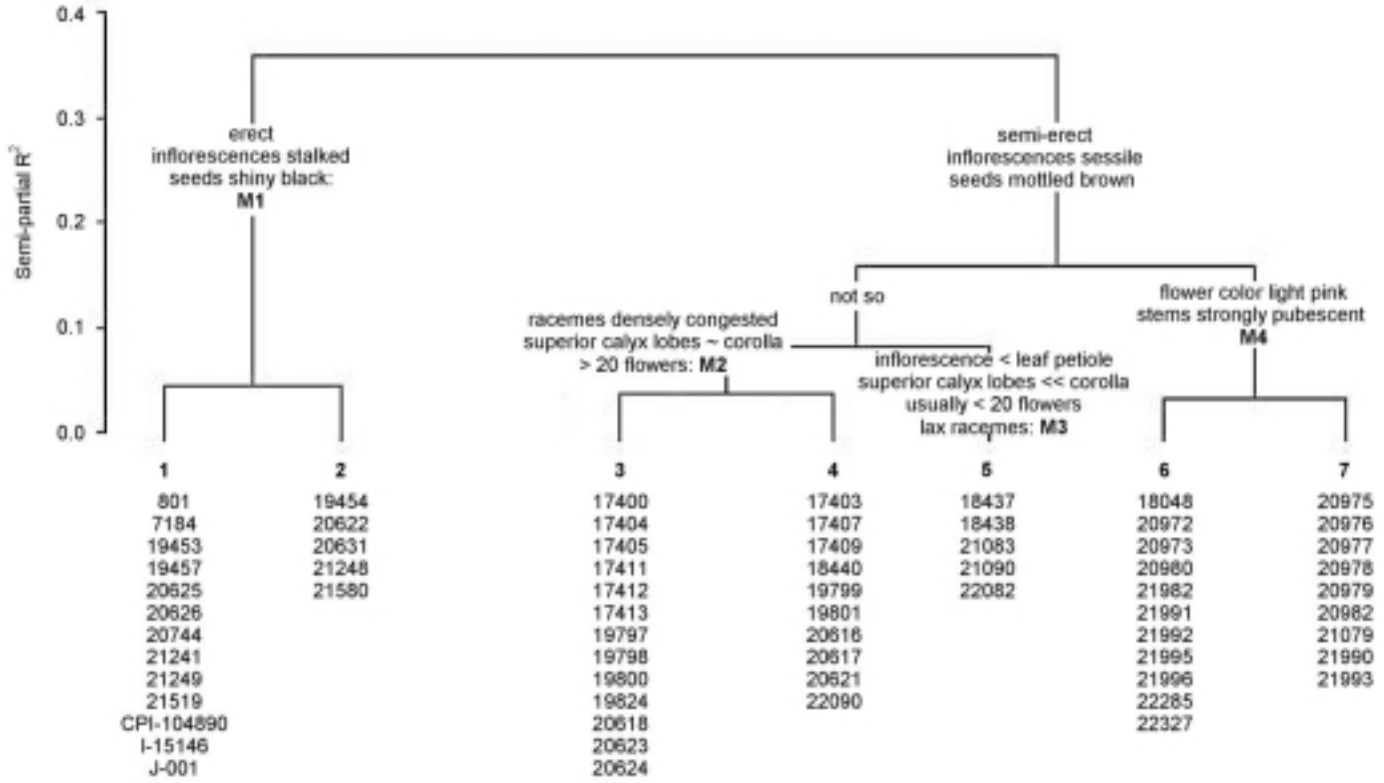

Figure 4.1 Cluster analysis dendrogram truncated at the 7-group level, identifying morphological groups of *Flemingia macrophylla*

Legend of following page:

[a] The original collection comprised 70 accessions, but CIAT 20065 and CIAT 22087 did not adapt; [b] D, dark pink; L, light pink; W, white; [c] B, shiny black; M, mottled brown; [d] G, glabrous to slightly pubescent; P, very pubescent; [e] CHN, China; CMR, Cameroon; COL, Colombia; CRI, Costa Rica; GHA, Ghana; IDN, Indonesia; IND, India; MYS, Malaysia; PNG, Papua New Guinea; THA, Thailand; HAW/USA, Hawaii/USA; VNM, Vietnam; [f] SEM, standard error of the mean.
* doubtful (see text)

Table 4.4 Growth, flowering and seed characteristics of a collection of 68 *Flemingia macrophylla* accessions grown at the CIAT-Quilichao Experimental Station, Colombia (Legend: see previous page)

Accession (CIAT No.)[a]	Plant height (cm)	Days to flower-ing	Inflorescence length (mm)	Peduncle length (mm)	Flower colour[b]	Seed colour[c]	Leaf area (cm^2)	Petiole length (mm)	Stem pubes-cence[d]	Seed yield (g $plant^{-1}$)	1000-seed weight (g)	Morpho-type (Fig. 4.1)	Geographic origin[e]
801	260	299	75	25	L	B	93	49	G	55	19	M1	unknown
7184	220	263	65	50	L	B	98	64	G	0	18	M1	unknown
17400	103	215	35	0	D	M	103	52	G	11	15	M2	THA
17403	115	259	55	0	D	M	104	42	G	18	22	M2	THA
17404	123	229	40	0	D	M	109	41	G	15	16	M2	THA
17405	110	199	55	0	D	M	134	42	G	16	17	M2	THA
17407	140	244	45	0	D	M	96	40	G	10	21	M2	THA
17409	150	273	50	0	D	M	138	64	G	12	17	M2	MYS
17411	120	232	40	0	D	M	110	45	G	34	16	M2	MYS
17412	153	258	55	0	D	M	124	75	G	0	16	M2	MYS
17413	143	245	45	0	D	M	78	31	G	44	16	M2	MYS
18048	55	249	30	0	L	M	57	26	P	0	14	M4	CHN
18437	110	303	40	0	D	M	106	78	G	-	14	M3*	IDN
18438	130	292	35	0	D	M	59	37	G	3	12	M3	THA
18440	137	244	35	0	D	M	106	46	G	18	22	M2	THA
19453	240	279	75	35	L	B	88	45	G	12	17	M1	PNG
19454	250	256	80	25	L	B	150	61	G	115	21	M1	PNG
19457	253	275	70	25	L	B	78	41	G	0	20	M1	PNG
19797	100	208	40	0	D	M	216	80	G	110	15	M2	IDN
19798	103	225	45	0	D	M	106	56	G	15	14	M2	IDN
19799	107	265	50	0	D	M	65	50	G	0	19	M2	IDN
19800	133	242	40	0	D	M	111	52	G	0	16	M2	IDN
19801	155	263	50	0	D	M	109	63	G	32	15	M2	IDN
19824	140	268	50	0	D	M	55	43	G	0	17	M2	IDN
20616	137	304	50	0	D	M	120	67	G	52	19	M2	IDN
20617	163	301	45	0	D	M	105	61	G	0	17	M2	IDN
20618	130	204	45	0	D	M	108	55	G	84	17	M2	IDN
20621	177	299	45	0	D	M	109	57	G	53	19	M2	IDN
20622	270	256	80	25	L	B	133	44	G	118	20	M1	IDN
20623	102	227	35	0	D	M	107	68	G	51	15	M2	IDN
20624	130	239	50	0	D	M	79	37	G	4	17	M2	IDN
20625	280	288	70	25	L	B	174	40	G	12	22	M1	IDN
20626	273	285	70	40	L	B	90	41	G	33	19	M1	IDN

20631	213	180	75	25	L	B	136	44	G	100	19	M1	IDN
20744	263	273	75	35	L	B	104	40	G	55	20	M1	COL
20972	40	199	35	0	L	M	55	33	P	27	17	M4	CHN
20973	50	247	40	0	L	M	52	30	P	19	17	M4	CHN
20975	103	268	45	0	L	M	146	42	P	18	17	M4	CHN
20976	110	246	35	0	L	M	71	48	P	11	15	M4	CHN
20977	120	291	50	0	L	M	66	38	P	0	15	M4	CHN
20978	140	256	60	0	L	M	94	47	P	15	16	M4	CHN
20979	73	256	28	0	L	M	92	43	P	5	16	M4	CHN
20980	113	202	40	0	L	M	72	43	P	55	17	M4	CHN
20982	110	265	40	0	L	M	92	53	P	18	15	M4	CHN
21079	73	270	35	0	L	M	89	53	P	30	15	M4	THA
21083	157	381	25	0	D	M	76	49	G	0	12	M3	THA
21087	170	-	-	-	-	M	88	48	G	-	24	-	THA
21090	163	355	23	0	D	M	73	47	G	4	15	M3	THA
21241	273	263	80	35	L	B	101	51	G	0	19	M1	COL
21248	257	290	75	20	L	B	156	59	G	110	19	M1	GHA
21249	230	277	65	25	L	B	104	51	G	0	18	M1	unknown
21519	280	269	75	25	L	B	147	65	G	9	18	M1	HAW/USA
21529	277	312	95	25	L	B	100	55	G	0	18	M1	IDN
21580	287	261	65	35	L	B	161	61	G	140	21	M1	CMR
21982	40	206	28	0	L	M	45	27	P	0	11	M4	VNM
21990	50	258	30	0	L	M	71	44	P	30	16	M4	VNM
21991	50	221	28	0	L	M	88	63	P	67	16	M4	VNM
21992	55	228	28	0	L	M	81	41	P	13	13	M4	VNM
21993	47	238	35	0	L	M	87	46	P	20	14	M4	VNM
21995	43	199	43	0	L	M	31	32	P	-	20	M4	VNM
21996	50	242	23	0	L	M	44	42	P	25	13	M4	VNM
22082	140	388	18	0	W	M	45	40	G	0	08	M3	THA
22090	63	248	55	0	D	M	56	41	G	50	22	M2	THA
22285	53	199	35	0	L	M	71	43	P	59	15	M4	VNM
22327	73	279	35	0	L	M	64	37	P	32	18	M4	VNM
CPI-104890	167	266	70	35	L	B	77	44	G	44	20	M1	IND
I-15146	257	286	70	40	L	B	108	64	G	25	23	M1	CRI
J-001	257	261	75	35	L	B	107	52	G	81	18	M1	COL
Mean	148	259	50	9			97	49		40	17		
SEM[†]	9.2	4.8	2.2	1.8			4.1	1.4		4.9	0.4		

Table 4.5 Growth, flowering and seed characteristics of the four morphotypes of *Flemingia macrophylla* at the CIAT- Quilichao Experimental Station, Colombia

	Group M1 (*n* = 19)	Group M2 (*n* = 23)	Group M3 (*n* = 5)	Group M4 (*n* = 20)
Growth habit	erect	semi-erect	semi-erect	semi-erect
Plant height [cm, after 14 months]	167-287 (253)*	63-177 (129)	110-163 (140)	40-140 (73)
Plant diameter [cm, after 14 months]	170-230 (198)	157-280 (203)	150-230 (182)	90-173 (126)
Ratio height:diameter	1.3	0.6	0.8	0.6
Inflorescence	lax	dense, congested	lax	dense, congested
Flowers per inflorescence [no.]	> 20	> 20	< 20	> 20
Inflorescence length [mm]	65-95 (74)	35-55 (46)	18-40 (28)	23-60 (36)
Peduncle length [mm]	20-50	sessile	sessile	sessile
Flower colour	light pink	dark pink	dark pink or white	light pink
Length of superior calyx lobes	≅ corolla	≅ corolla	<< corolla	≅ corolla
Leaf area [cm^2]	77-174 (116)	55-216 (106)	45-106 (72)	31-146 (73)
Petiole length [mm]	40-65 (51)	31-80 (52)	37-78 (50)	26-63 (41)
Ratio inflorescence:petiole length	1.5	0.9	0.6	0.9
Stem and petiole pubescence	glabrous	glabrous	glabrous	very pubescent
Days to flowering [after sowing]	180-312	199-304	292-388	199-291
Days to pod setting [after sowing]	210-337	283-339	> 340	277-314
Seed colour	shiny black	brown-mottled	brown-mottled	brown-mottled
Average seed weight [g 1000-$seeds^{-1}$]	19	18	12	15
Seed yield [g $plant^{-1}$]	9-140	4-110	3-4	5-67

* mean values in ( )

Within morphotype M1 (erect plants), one subgroup comprising accessions CIAT 19454, CIAT 20622, CIAT 20631, CIAT 21248 and CIAT 21580 is further separated (Figure 4.1) due mainly to their larger leaves and 7-fold higher seed production. Within morphotype M2, a subgroup of ten accessions can be distinguished with higher plant height, later flowering date and lower seed yield. Within morphotype M4, two subgroups can be distinguished with nine and 11 accessions, respectively. The latter differ mainly due to their lower plant height and smaller leaves. They flowered on average four weeks earlier and had a two-fold higher seed yield. The ratio plant height:plant diameter allowed to separate these groups, and was particularly useful for the distinction of morphotypes M1 and M4, while the groupings of M2 and M3 somewhat overlapped (Figure 4.2).

Visual inspection in the field and of herbarium material indicated that CIAT 18437, which was grouped together with four other accessions as morphotype M3, should rather be classified as M2 due to its inflorescence characteristics (sessile, congested, with numerous dark pink flowers). Cluster analysis indicated that the grouping of this accession with four morphotype M3 accessions was due to its late flowering date (299 days), low flowering intensity (1.0) and low inflorescence:petiole length ratio (0.5), as compared to other M2 accessions.

Discussion

The morphological and phenological characterization led to the distinction of one erect and three semi-erect *F. macrophylla* morphotypes, differing in growth, flowering and seed characteristics. The ratio plant height:plant diameter proved to be a useful tool for separating the morphotypes (Figure 4.2) and could be a convenient attribute for the characterization of shrub growth habits in general. However, the ratio can be altered because growth habits can change when submitted to a cutting regime, so the use of a ratio is relative to the environment or agronomic management.

Morphotype seems to be at least partly related to geographic origin (Figure 4.3): For example, only nine of the 19 accessions with erect growth habit (M1) were collected in Asia, namely in India (CPI-104890), Indonesia (Sumatra: CIAT 20622, CIAT 20625, CIAT 20626 and CIAT 20631; Java: CIAT 21529), and Papua New Guinea (CIAT 19453, CIAT 19454, CIAT 19457). All other erect accessions are from other continents: Cameroon and Ghana in Africa; Colombia and Costa Rica in tropical America; and Hawaii in the Pacific. In contrast, all semi-erect accessions were collected in Southeast Asia.

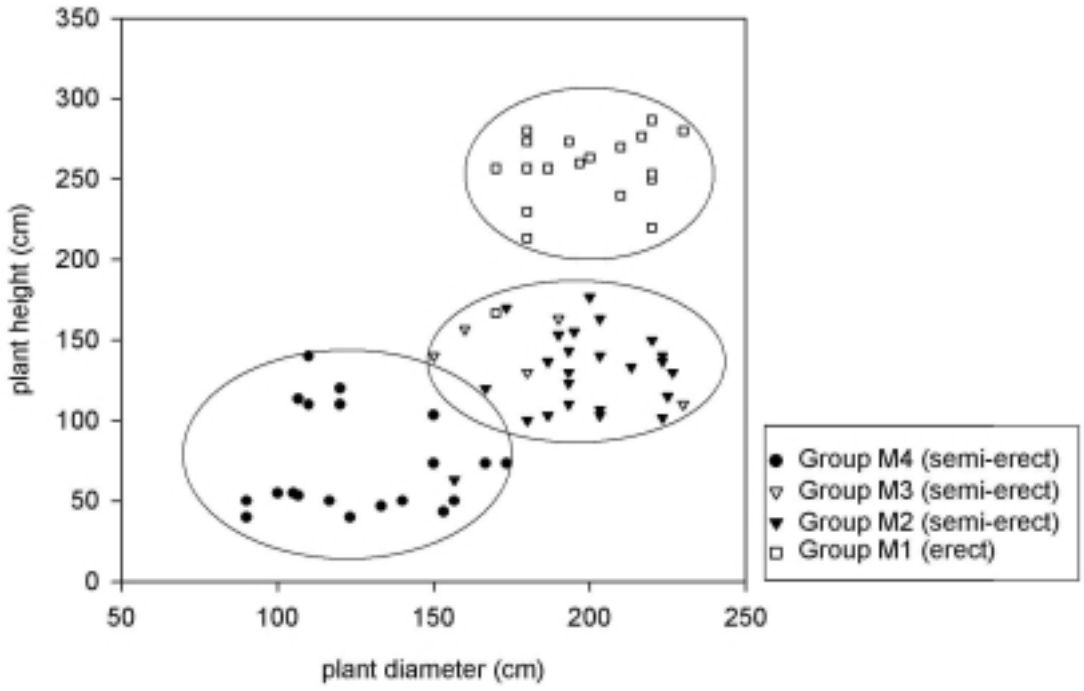

Figure 4.2 Relationship between plant height and diameter of accessions of four *Flemingia macrophylla* morphotypes grown at the CIAT-Quilichao Experimental Station, Colombia

Figure 4.3 Geographic origin of 60 accessions of four *Flemingia macrophylla* morphotypes collected in Southeast Asia

These accessions belonged to morphotype M2 (dark pink flowers, stems and petioles glabrous or slightly pubescent) and M3 (less than 20 dark pink or white flowers in very lax racemes, usually not exceeding the length of the petiole), and were collected in central Southeast Asia (Thailand, Malaysia, Sumatra/Indonesia). All accessions of morphotype M4 (light pink flowers, strongly pubescent stems and petioles) were collected in China and Vietnam, except CIAT 21079 which is from Thailand.

The great similarity of the non-Asian accessions in the collection with commercial material obtained from development projects in Southeast Asia (accessions CIAT 801 and CIAT 7184) in terms of morphological and phenological characteristics suggests that these materials might be derived from introduced Southeast Asian accessions. This would confirm other indications that *Flemingia macrophylla* was most probably introduced to Africa and America from Southeast Asia (L.J.G. van der Maesen, pers. communication 2005). Furthermore, in a molecular marker analysis of the *F. macrophylla* collection with random amplified polymorphic DNA (RAPD; Chapter 7), nine accessions were identified as genetically identical and thus as duplicates. These were CIAT 801, CIAT 7184 and CIAT 21249 without origin information; CIAT 21519 from Hawaii; CIAT 19454 and CIAT 19457 from Papua New Guinea; CIAT 21529 from Java Island; and CIAT 20622 and CIAT 20631 from North Sumatra. In the same study, the African accession CIAT 21248 (Ghana) was identified as a duplicate of CIAT 19453 from Papua New Guinea.

The pronounced differences among the four morphotypes identified in this study may imply taxonomic distinction. Usually, quantitative traits such as inflorescence and petiole length, leaf area and plant pubescence are highly variable since they are influenced by environment. Similarly, flower and seed colour vary greatly within taxa and even within accessions. Thus, large variability of any of these traits does not necessarily imply taxonomic differences, but can indicate genetic differences below the species level. In the present case, however, variability among morphotypes was also qualitative, e.g. stalked vs. sessile, and branched vs. unbranched inflorescences, in combination with markedly distinct ranges and ratios of quantitative traits.

Furthermore, some traits (e.g. congested vs. lax inflorescences, superior calyx lobes:corolla length ratio, inflorescence:petiole length ratio) were explicitly chosen due to their taxonomic relevance and their use in floras distinguishing *Flemingia* species and subspecies (e.g., Merrill 1910; Thuân 1979; Verdcourt 1979). It is therefore hypothesized that the differences detected among morphotypes reflect not only natural intraspecific variation, but have also taxonomic implications. The molecular marker analysis with RAPD provides further evidence supporting this hypothesis and demonstrates that more than 75% of the genetic diversity is due to genetic differentiation among morphotypes (Chapter 7). The flowering times of the four morphotypes, however, overlap widely, indicating that no biological (temporal) outcrossing barrier exists between them. This, together with the wide overlap of their geographic occurrence, suggests that they should be treated as botanical varieties or subspecies, and not as separate species.

To date, different morphotypes have only been reported for the tropical tree legumes *Leucaena leucocephala* (Dommergues *et al.* 1999) and *Calliandra calothyrsus* (Chamberlain 1998; Macqueen 2001), and recently for the tropical shrub legume *Desmodium velutinum* (N. Rivas, personal communication 2005). Furthermore, published morphological characterizations of tropical tree and shrub legumes in general are rare. Morphological characterizations such as the one presented here can offer valuable information of taxonomic relevance which usually cannot be obtained by mere analysis of dried herbarium material. Furthermore, the characterization of isolated plants of the same age provides morphological details which would be difficult to obtain when observing a plant in its natural habitat and vegetation community.

To date, little is known about factors responsible for flowering induction in the indeterminate *F. macrophylla*. The related pigeonpea *Cajanus cajan* (L.) Millsp. is a short-day species (Maesen 1986), and its photoperiod sensitivity varies among genotypes. Generally, the main external factors affecting flowering in tropical legumes are photoperiod and moisture stress (Schultze-Kraft and Keller-Grein 1999), and recently, a combination of cumulative changes in day length and advancing sunrise/sunset time has been proposed as the main factor

responsible for flowering induction at latitudes between 6° N and 6° S, due to the fact that these changes are greatest at the equinoxes (Borchert *et al.* 2005). In *Flemingia macrophylla*, the principal flowering peak after sowing and after cutting occurred in December, i.e. a few weeks after the dry season (July to September) as well as after the autumn equinox (mid September). It is therefore suggested that decreasing day length, advancing sunset time or water deficit stress, but probably a combination of these factors, affect flowering in *F. macrophylla*. Experimental treatments are needed to test the phenological response of *F. macrophylla*, particularly in view of the limited scope of the present study (one site, one year, differing latitudes and agronomic management not represented).

Conclusions

The morphological characterization of *Flemingia macrophylla* clearly identified four different morphotypes and provided evidence for taxonomic implications. Further morphological and molecular marker studies are needed to clarify the taxonomic status of the four morphotypes (species, subspecies, botanical varieties) and the relationship among them. These include the comprehensive analysis of herbarium specimens, focusing in particular on inflorescence characteristics (e.g. ramification of inflorescence, stalk length, number of nodes per inflorescence and of flowers per node, length of calyx lobes, pubescence of corolla, petioles and pods, presence/absence of glands) of a larger sample of accessions, including comprehensive analysis of herbarium specimens.

The ratio plant height:plant diameter proved to be a useful tool for separating the morphotypes and could be a convenient attribute for the characterization of shrub growth habits in general. It must be kept in mind, however, that the ratio can be altered and growth habits can change when plants are submitted to a cutting regime.

This is the first study of flowering behaviour of *Flemingia macrophylla*. Results suggest changes in day length and sunset time as well as moisture stress as main external factors affecting flowering induction in this species. In view of the limited scope of the present study (one site, one year, differing latitudes and agronomic management not represented), the phenological response of *F. macrophylla* should be tested in experimental treatments. Furthermore, comprehensive agronomic and forage quality evaluations should be conducted in order to assess the variability within morphotypes, identify differences among them and determine whether any of them is particularly promising for further evaluation.

Chapter 5

Agronomic and forage quality evaluation of the *Flemingia macrophylla* world collection

Abstract

The diversity of the drought-tolerant, tropical multipurpose shrub legume *Flemingia macrophylla* (Willd.) Merrill was assessed in terms of agronomic and forage potential. Large variability of dry matter (DM) production (1 to 636 g plant^{-1} and 1 to 285 g plant^{-1} in the wet and dry seasons, respectively), *in vitro* DM digestibility (IVDMD, 283 to 581 g kg^{-1}) and crude protein content (140 to 252 g kg^{-1}) was measured. Plant height ranged from 15 to 162 cm, and regrowth capacity from 3 to 88 regrowth shoots plant^{-1}. CIAT 18437, CIAT 21083 and CIAT 21090 were identified as promising accessions in terms of productivity and digestibility. Their IVDMD was up to 507 g kg^{-1} in the dry and up to 541 g kg^{-1} in the wet season. DM yield and IVDMD were superior to control CIAT 17403. An exploratory cafeteria grazing trial showed that *F. macrophylla* was palatable in the dry but not consumed in the wet season. Further development, such as palatability studies with a selected set of accessions, can make *Flemingia* a valuable feed alternative.

Keywords: agronomic evaluation, cafeteria trial, dry season feed, *Flemingia macrophylla*, forage quality, multipurpose legumes, palatability, tannins, tropical legumes

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Introduction

Flemingia macrophylla (Willd.) Merrill (syn. *F. congesta, Moghania macrophylla*) is a drought-tolerant, multipurpose shrub legume growing up to 4 m high. It is especially suited to low-input smallholder production systems in the sub-humid and humid tropics: the species enhances soil fertility and is used for a number of purposes such as dry season forage supplementation, live soil cover or mulch, erosion barrier hedges, shade-providing shrubs in young coffee and cocoa plantations, and as firewood (Andersson *et al.* 2002). There are also reports on several other uses such as dye, for medicinal purposes and nematode control (Budelman and Siregar 1997; Banful *et al.* 2000).

In studies conducted in Cambodia and Vietnam, *F. macrophylla* proved to be very suitable as a potential perennial forage shrub in association with cassava, incrementing cassava root and foliage yield, restoring soil fertility and reducing erosion (Preston *et al.* 2000; Tien Dung *et al.* 2005). Its particular advantages are vigour, leafiness, a wide range of soil adaptation including very acid, low-fertility soils, drought tolerance, excellent coppicing capacity and regrowth after cutting, and slow leaf decomposition. The species' main limitation is low nutritive value in terms of digestibility because of high tannin content combined with low palatability to cattle (Thomas and Schultze-Kraft 1990; Jackson *et al.* 1996; Barahona *et al.* 1997).

In the previous chapter, the morphological and phenological diversity in the world collection of 69 accessions classified in germplasm databases as *F. macrophylla* was assessed (Chapter 4, Andersson *et al.* 2005b). The objective of the study presented in the present chapter was to i) assess the variability in the world collection in terms of agronomic and forage quality parameters, ii) identify promising accessions for further evaluation, and iii) assess the species' agronomic and forage potential, its opportunities and limitations. Therefore, agronomic evaluations and a forage quality characterization assessing *in vitro* digestibility and crude protein content were carried out. An exploratory cafeteria trial was conducted in order to assess relative palatability.

Materials and methods

Selection of the accessions

The collection, assembled mainly from Southeast Asia, comprises 69 accessions classified in germplasm databases (e.g., the Systemwide Information Network on Genetic Resources, SINGER of the Consultative Group on International Agricultural Research, CGIAR) as *Flemingia macrophylla* (Willd.) Merrill held in the Genetic Resources Unit of the International Centre for Tropical Agriculture (CIAT).

According to results from a morphological characterization (Chapter 4), the collection was classified into four morphotypes (M, see Table 5.1), distinguishing one erect (M1: long, stalked racemes with numerous light pink flowers and black seeds) and three semi-erect (M2, M3 and M4: shorter, unstalked (sessile) racemes with dark or light pink (rarely white) flowers and mottled, brown seeds) morphotypes. Accession CIAT 17403 ("Chumphon" in Southeast Asia, Horne and Stür 1999) had frequently been used in other experiments (Thomas and Schultze-Kraft 1990; Cano *et al.* 1994) because of its good dry matter production and was therefore chosen as a control. CIAT 21092 (an accession of *Flemingia stricta* Roxb.) was included as an additional reference accession due to its high vigour and remarkably great leaf size.

Evaluation site

The research was conducted at the CIAT-Quilichao Experimental Station in the Cauca department, Colombia (03°06' N, 76°31' W; 990 m asl; average annual temperature 23 °C). Average annual rainfall is 1800 mm, distributed in a bimodal pattern with two generally well pronounced rainy seasons from March to June and from September to December. Rainfall during the establishment period (April–December 2000) was 1456 mm, and 1345 mm and 1265 mm during the two subsequent experiment years (Figure 5.1). The soil of the experimental plots is an acid Ultisol (pH = 5.3) with 76% Al saturation, medium P (6 ppm Bray II) and high organic matter content (7.4%). Seeds were sown in Jiffy pots, inoculated with *Bradyrhizobium* strain CIAT 4099, and seedlings transplanted from the greenhouse to the field after six weeks. A Randomised Complete Block design with three replications was used, each arranged as 9-plant single-row plots with a distance of 1 m between plants and 1.5 m between rows. Four weeks after transplanting, fertilizer was applied as 40:50:20 kg ha^{-1} of P:K:Mg, Mo as 500 g ha^{-1} to single plants to ensure good establishment.

Agronomic evaluation

Six months after transplanting to the field (November 2000, wet season) plant height and diameter (cm) were measured, vigour (rating: 0 = plant dead, 5 = very vigorous) was rated, and the incidence of pests and diseases (rating: 0 = not present, 5 = severe damage) and of mineral deficiencies (rating: 0 = no deficiency, 5 = severe deficiency) was assessed. A standardization cut was done seven months after transplanting at a height of 15 cm for prostrate accessions and 30 cm for all others. Subsequently, plants were cut at eight-week intervals during 24 months. Plant height, diameter, and dry matter (DM) production (g $plant^{-1}$) of the three central plants were measured during the wet and dry season, as well as regrowth capacity (number of regrowth shoots), and the incidence of pests, diseases and mineral deficiencies was assessed. For DM determination, the samples were separated into edible and non-edible fractions, according to earlier observations of the diameter of branches consumed by cattle (leaves and stems with diameter < 10 mm and > 10 mm, respectively), oven-dried at 70 °C for 72 hours and subsequently edible, non-edible and total DM production (g $plant^{-1}$) was calculated.

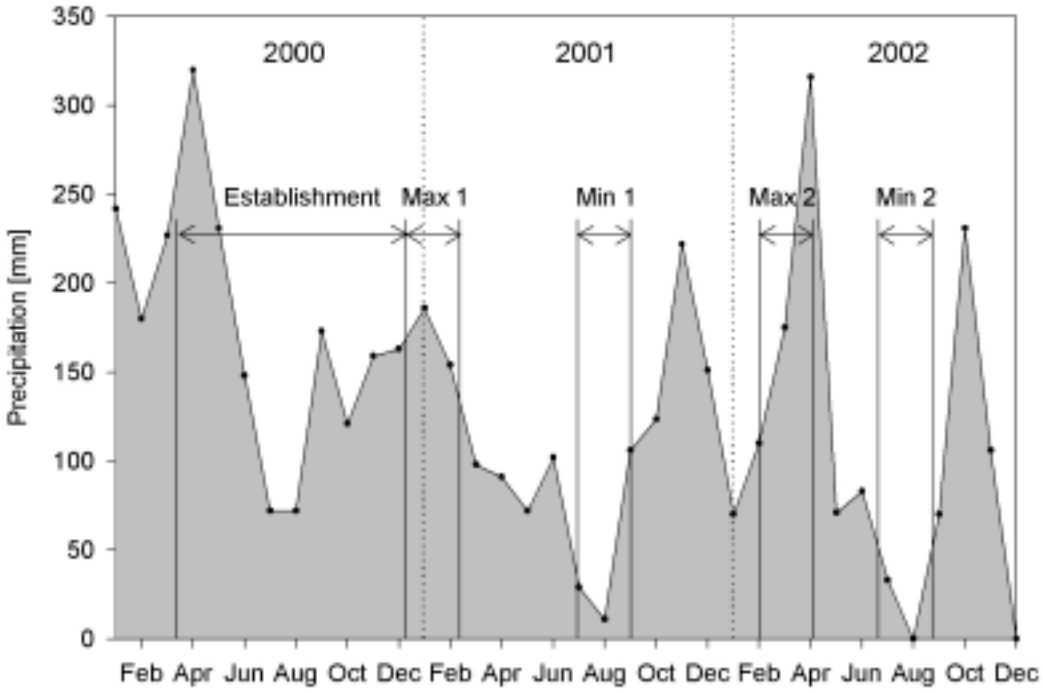

Figure 5.1. Monthly rainfall and wet and dry seasons at the trial location during the three study years. Evaluation cuts were conducted at the end of each season

Forage quality characterization and cafeteria grazing trial

Leaves for quality characterization were harvested when plants were cut for the agronomic evaluations. The samples were freeze-dried and ground to pass a 1-mm screen. The *in vitro* dry matter digestibility (IVDMD) was estimated using the two-stage technique (Tilley and Terry 1963) modified by Harris (1970). Total nitrogen (N) was determined by the Kjeldahl procedure (AOAC, 2003) and multiplied by 6.25 to obtain an estimate of crude protein (CP).

At the end of the experimental period, an exploratory cafeteria grazing trial using cattle (Schultze-Kraft *et al.* 1989) was carried out in 2003, including the *F. stricta* reference accession CIAT 21092. For this purpose, the site of the agronomic evaluation (Randomized Complete Block design with three replications) was fenced, so that cattle could chose among all genotypes. Three young crossbred steers (body weight 260 kg), raised on a diet of *Bracchiara* before the experiment, were introduced in the site after eight weeks of regrowth, once during a dry and once during a wet season. Due to availability problems of cattle, different animals had to be used in these seasons. The animals were exposed to the new forage directly during a seven-day period, without prior adjustment. No other feed was offered. The behaviour of each animal (eating, walking, resting, ruminating) was recorded every five minutes from 9 to 12 a.m. and 1 to 3 p.m., recording the accession number in the case of consumption.

Statistical analysis

Principal component analysis (PCA) was applied on the agronomic and forage quality attributes of the 69 *F. macrophylla* accessions evaluated in two wet and two dry seasons and correlations were calculated. When the correlation coefficient (r) between two variables was greater than 0.7, then only one of these was included in the subsequent cluster analysis. Due to its agronomic importance, DM yield was included for both the wet and dry season although highly correlated with each other ($r > 0.9$).

Incidences of pests, diseases and mineral deficiencies were not significant and therefore were excluded from the cluster analysis. Cluster analysis was performed using Ward's Method (Ward 1963). Based on the results from the PCA analysis, DM production, regrowth capacity, IVDMD and CP content were selected as the most appropriate parameters for the identification of groups of agronomic interest.

Data on plant height, plant diameter, regrowth capacity and DM production were subjected to analysis of variance (GLM-ANOVA procedure) using a model containing seasons, replications (blocks), accessions and experimental error as the main sources of variation. Laboratory duplicate or triplicate samples were averaged for each experimental unit prior to the analysis of variance so that a pooled estimate of sampling variance was not obtained. Means were separated at $P < 0.05$ using the Ryan-Einot-Gabriel-Welsch Multiple Range test. For the grazing trial, a relative palatability index (RPI) was calculated by dividing the number of times a given accession was consumed by the number of times the accession should be expected to be consumed, if all accessions were of equal palatability (Schultze-Kraft *et al.* 1989). Accessions were classified as follows: very low palatability (RPI < 1.0); low palatability (RPI 1.0-2.0); moderate palatability (RPI > 2.0). All analysis were performed using the SAS program package version 8.2 (SAS Institute Inc. 1999).

Results

All accessions adapted well to the experimental site, except CIAT 20065 and CIAT 22087. In general, initial plant establishment was slow and manual weeding was required. After six months, plant height ranged from 25 to 191 cm (average 104 cm) and plant diameter from 44 to 164 cm (average 110 cm). Plant vigour was high (average 4.0) and there was virtually no incidence of pests, diseases or mineral deficiencies (averages of 1.3, 0.2 and 0.0, respectively).

After the standardization cut, large variability of plant height, regrowth capacity, DM production, IVDMD, and CP content was detected among accessions (Table 5.1 and Figure 5.2). Plant height ranged from 21 to 156 cm in the wet and from 15 to 127 cm in the dry season, with an average of 80 and 62 cm, respectively.

Table 5.1 Plant height, regrowth capacity, dry matter (DM) yield, *in vitro* dry matter digestibility (IVDMD) and crude protein (CP) content of a collection of 69 *Flemingia macrophylla* accessions in the wet and dry season at the CIAT-Quilichao Experimental Station, Colombia

Accession (CIAT No.)	Morpho type*	Plant height (cm)		Regrowth (no. of shoots per plant)		DM yield (g plant^{-1})		IVDMD (g kg^{-1})		CP (g kg^{-1})	
		wet	dry	wet	dry	wet	dry	wet	dry	wet	dry
801	M1	138	105	26	30	344	180	426	414	237	232
7184	M1	127	105	30	38	357	266	402	408	219	227
19453	M1	109	81	17	20	162	93	431	392	206	228
19454	M1	121	89	21	24	243	134	450	384	202	206
19457	M1	119	105	23	27	215	175	398	379	211	218
20622	M1	156	127	26	32	323	223	469	422	226	216
20625	M1	130	114	22	28	305	185	465	405	231	224
20626	M1	123	102	25	34	269	214	447	443	223	198
20631	M1	118	101	22	26	321	204	461	437	213	214
20744	M1	127	105	27	28	296	180	471	416	228	224
21241	M1	151	112	25	27	362	195	411	476	214	227
21248	M1	143	109	31	34	406	248	393	400	230	200
21249	M1	148	106	31	37	508	257	442	386	222	205
21519	M1	139	109	24	31	359	193	440	423	225	212
21529	M1	144	113	31	30	420	200	436	392	223	218
21580	M1	153	110	40	32	586	272	419	429	203	214
CPI-104890	M1	111	88	33	38	376	196	397	395	226	226
I-15146	M1	115	94	23	29	343	179	439	407	234	229
J-001	M1	134	106	30	32	315	175	440	402	232	231
17400	M2	70	50	31	34	236	108	402	347	220	226
17404	M2	65	54	30	35	188	113	395	365	213	213
17405	M2	64	53	30	31	188	131	424	375	213	209
17407	M2	85	71	34	41	298	181	393	382	196	199
17409	M2	61	54	36	37	295	157	391	369	197	214
17411	M2	65	51	31	38	217	173	417	368	212	203
17412	M2	82	69	39	42	267	167	414	376	198	212
17413	M2	71	52	32	38	212	127	411	345	205	212
18440	M2	64	53	32	33	222	112	339	327	209	208
19797	M2	63	50	22	22	183	105	428	383	210	210
19798	M2	69	54	25	30	228	153	446	419	206	212
19799	M2	70	56	26	27	191	122	423	389	205	214
19800	M2	75	65	34	37	208	150	378	362	203	213
19801	M2	87	71	38	43	251	151	398	336	215	216
19824	M2	71	58	34	38	237	164	427	394	213	227
20616	M2	79	62	37	36	292	149	385	387	209	223
20617	M2	84	65	28	30	210	123	343	332	199	213
20618	M2	83	60	32	31	209	136	370	350	211	212
20621	M2	79	61	30	30	142	118	367	379	214	225
20624	M2	71	59	32	37	269	177	356	344	205	207
22090	M2	46	37	12	11	36	10	390	307	169	175
18437	M3	61	53	38	40	239	159	512	453	219	226
18438	M3	82	50	46	33	204	84	543	495	217	205
21083	M3	115	90	42	42	317	145	495	507	227	183
21090	M3	107	79	63	54	520	201	541	467	226	178
22082	M3	87	66	67	59	274	116	503	444	202	199
18048	M4	39	25	25	21	55	26	451	408	201	199

Accession (CIAT No.)	Morpho type*	Plant height (cm)		Regrowth (no. of shoots per plant)		DM yield (g plant^{-1})		IVDMD (g kg^{-1})		CP (g kg^{-1})	
		wet	dry	wet	dry	wet	dry	wet	dry	wet	dry
20975	M4	55	40	39	44	134	71	490	436	204	190
20976	M4	46	41	25	25	66	59	455	411	195	199
20977	M4	35	25	9	10	18	12	458	381	186	202
20978	M4	53	40	23	20	64	29	475	399	206	197
20979	M4	57	38	42	40	142	78	415	369	208	194
20980	M4	50	39	29	29	94	46	458	395	202	181
20982	M4	55	44	28	28	89	57	449	421	197	189
21079	M4	54	34	44	38	168	63	413	378	204	187
21991	M4	35	27	26	24	64	29	429	340	219	189
21992	M4	36	26	28	27	66	33	475	415	198	172
21993	M4	45	35	45	36	117	59	431	401	198	191
22285	M4	50	41	50	43	144	62	417	324	207	175
22327	M4	44	29	32	27	76	44	512	374	196	178
20972	M4	31	20	32	33	62	36	424	418	224	193
20973	M4	32	24	17	20	66	26	365	367	192	199
21982	M4	25	17	39	31	98	30	453	381	209	211
21990	M4	45	29	55	45	117	51	373	373	190	176
21995	M4	38	26	34	30	77	27	422	333	203	193
21996	M4	23	21	14	15	25	14	446	362	214	179
20065	-	21	15	4	5	5	2	406	454	201	225
21087	-	69	49	45	44	144	73	481	436	206	206
22087	-	28	21	17	16	44	14	413	400	187	172
17403	Control	75	61	32	36	262	153	415	361	216	211
21092	(*F. stricta*)	84	64	23	26	214	136	505	492	184	193
Mean		80	62	31	32	215	122	431	395	209	205
SEM		4.6	3.7	1.7	1.4	16.2	9.0	5.2	5.0	1.6	2.0

Data of two cuts per season after eight weeks of regrowth

* Morphotypes according to Chapter 4: M1, erect accessions (stalked racemes with numerous light pink flowers and black seeds); M2, semi-erect accessions with dark pink flowers in densely congested racemes and glabrous or slightly pubescent stems and petioles; M3, semi-erect accessions with less than 20 dark pink or white flowers in very lax racemes which usually do not exceed the length of the petiole; M4, semi-erect accessions with light pink flowers in densely congested racemes and strongly pubescent stems and petioles

Plant diameter varied from 21 to 131 cm in the wet and from 17 to 94 cm in the dry season, the averages being 92 and 67 cm, respectively. Regrowth ranged from 4 to 67 regrowth shoots plant^{-1}, and the incidence of pests, diseases and mineral deficiencies was again insignificant (average ratings 0.5, 0.1 and 0.0, respectively). Total DM production ranged from 5 to 586 g plant^{-1} (average 215 g plant^{-1}) in the wet and from 2 to 272 g plant^{-1} (average 122 g plant^{-1}) in the dry season. On average, 99% of total DM was edible and non-edible DM was produced by 17 erect accessions only in the first evaluation cut (wet season 1, see Figure 5.1). Therefore, from now on whenever referring to "DM production" "total DM production" is meant.

IVDMD varied from 307 to 543 g kg^{-1}, and CP content from 169 to 237 g kg^{-1}, with no differences between wet and dry season. Genotypes differed ($P < 0.01$) for plant height and diameter, regrowth, DM production and IVDMD. Season influenced DM production which was significantly higher (1.8-fold, $P < 0.01$) in the wet than in the dry season. Dry matter production was significantly correlated with plant height ($r = 0.81$, $P < 0.01$) and diameter ($r = 0.82$, $P < 0.01$) for both seasons. No genotype x season interaction ($P > 0.05$) was detected.

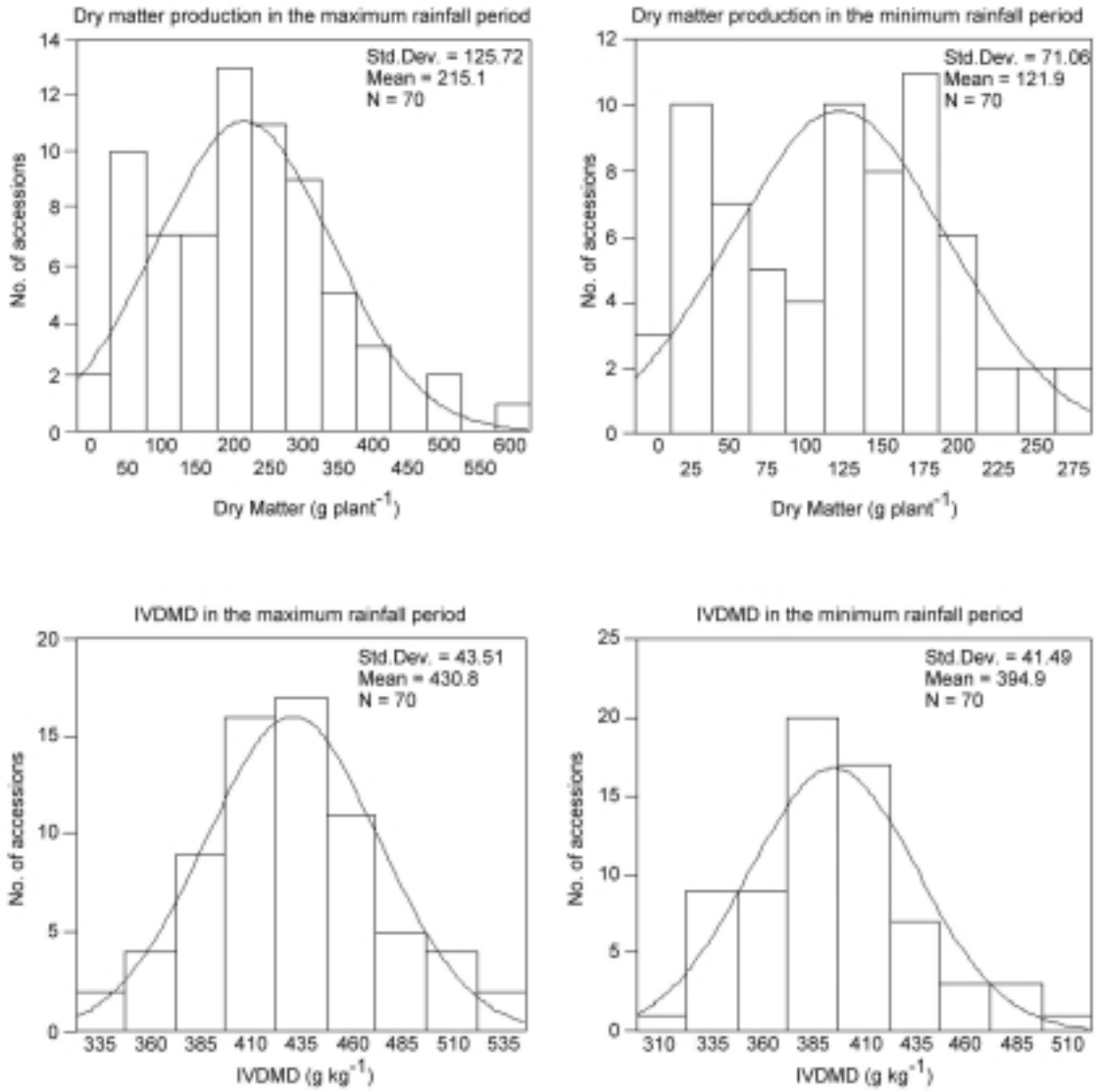

Figure 5.2 Dry matter production and *in vitro* dry matter digestibility (IVDMD) of 69 *Flemingia macrophylla* accessions and one *F. stricta* in the wet and dry season

The cluster analysis dendrogram was truncated at the 7-group level, explaining 72% of the total variation (Figure 5.3). The resulting clusters were classified as follows according to their group averages (Table 5.2). Plant height: low (< 0.50 m), moderate (0.50 to 0.99 m), high (≥ 1.00 m); regrowth capacity: low (< 20 shoots plant^{-1}), moderate (20 to 39 shoots plant^{-1}), good (≥ 40 shoots plant^{-1}); DM production: low (< 100 g plant^{-1}), moderate (100 to 199 g plant^{-1}), high (≥ 200 g plant^{-1}); IVDMD: low (< 400 g kg^{-1}), moderate (400 to 449 g kg^{-1}), high (≥ 450 g kg^{-1}); CP: low (< 170 g kg^{-1}), moderate (170 to 204 g kg^{-1}), high (≥ 205 g kg^{-1}).

Group 1 is conformed by 19 accessions with the lowest IVDMD values of the whole collection (391 g kg^{-1} in the wet and 362 g kg^{-1} in the dry season). DM production was 234 g plant^{-1} in the wet and 145 g plant^{-1} in the dry season. Group 2 is composed of six accessions similar to those of Group 3 in forage quality, but with lower DM yields (187 g plant^{-1} and 116 g plant^{-1}, respectively), and lower plant height. Group 3 contains 16 accessions with the highest DM production (370 g plant^{-1} in the wet, 210 g plant^{-1} in the dry season) and highest CP content (224 g kg^{-1}) of the whole collection, and the second highest IVDMD (435 g kg^{-1} in the wet, 416 g kg^{-1} in the dry season). Group 4 contains seven accessions with the highest digestibility values (509 g kg^{-1} in the wet and 463 g kg^{-1} in the dry season) and high DM production (265 g plant^{-1} in the wet and 121 g plant^{-1} in the dry season).

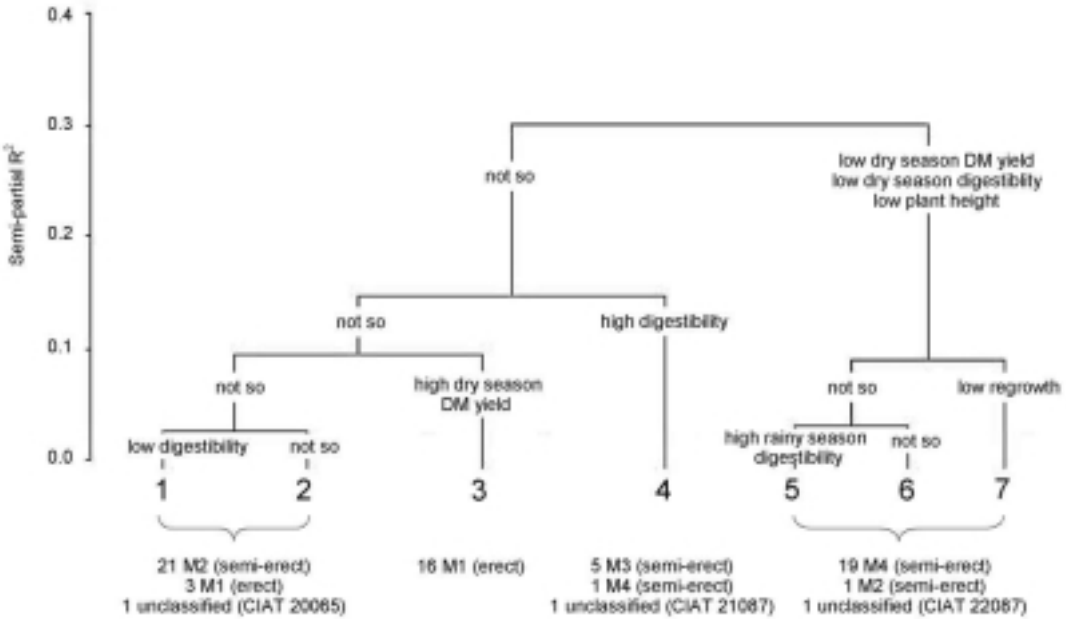

Figure 5.3 Cluster analysis dendrogram truncated at the 7-group level, identifying agronomic groups of *Flemingia macrophylla*. The morphotype (see Table 5.1) is given below the group numbers

Table 5.2 Identification of *Flemingia macrophylla* accessions of agronomic interest

Group	Agronomic characteristics	Accession (CIAT No.)	Prevailing morphotype*
1	intermediate regrowth capacity, intermediate to high DM yield, low IVDMD, high CP content	17400, 17403, 17404, 17405, 17407, 17409, 17411, 17412, 17413, 18440, 19457, 19800, 19801, 19824, 20616, 20617, 20618, 20621, 20624	M2
2	like Group 5, but lower DM yield	19453, 19454, 19797, 19798, 19799, 20065	M2
3	high DM yield and CP content, intermediate regrowth capacity and IVDMD	801, 7184, 20622, 20625, 20626, 20631, 20744, 21241, 21248, 21249, 21519, 21529, 21580, CPI-104890, I-15146, J-001	M1
4	high regrowth capacity and IVDMD, intermediate to high DM yield and CP content	18437, 18438, 20975, 21083, 21087, 21090, 22082	M3
5	low DM yield, intermediate regrowth capacity, low to high IVDMD, intermediate to high CP content	18048, 20972, 20976, 20978, 20980, 20982, 21982, 21991, 21992, 21995, 21996, 22327	M4
6	low to intermediate DM yield and IVDMD, intermediate CP content, high regrowth capacity	20979, 21079, 21990, 21993, 22285	M4
7	low regrowth capacity and DM yield, low to intermediate IVDMD, intermediate CP content	20973, 20977, 22087, 22090	M4

* see Table 5.1

Three of these accessions (CIAT 18437, CIAT 21083 and CIAT 21090) were superior to control accession CIAT 17403 in terms of digestibility and DM yield. Group 5 comprises 12 accessions with relatively high digestibility and CP content in the wet season (IVDMD 454 g kg^{-1}, CP 205 g kg^{-1}), but low in the dry season (IVDMD 388 g kg^{-1}, CP 190 g kg^{-1}). Plant height is very low, as well as DM production (70 g $plant^{-1}$ in the wet, 36 g $plant^{-1}$ in the dry season). Group 6 contains five accessions with conspicuously low DM production, IVDMD and CP content. Group 7 is made up of four accessions and differs from Group 6 mainly in lower regrowth capacity (9-20 vs. 36-54 regrowth shoots $plant^{-1}$) and CP content (184 vs. 201 g kg^{-1}).

Clustering results in a relatively clear separation of the four morphotypes described in Chapter 4 (Figure 5.3). Sixteen of the 19 erect accessions classified as morphotype M1 cluster together in the agronomic Group 3. All accessions of the semi-erect morphotype M2 (dark pink flowers in densely congested racemes and glabrous or slightly pubescent stems and petioles), except CIAT 22090, fall into the agronomic Groups 1 and 2, together with the remaining three erect accessions of morphotype M1.

Table 5.3 Palatability and other agronomic and forage quality characteristics of the ten *Flemingia macrophylla* accessions most frequently consumed in the dry season and the ten accessions with the highest *in vitro* dry matter digestibility (IVDMD)

Accession (CIAT No.)	Palatability			Other		
	Total Freq[a]	Freq day^{-1}	RPI[b]	IVDMD[c] (g kg^{-1})	Agron. group[d]	Morpho-type[e]
17409	21	3.0	4.41	369	1	M2
20616	20	2.9	3.90	387	1	M2
17407	19	2.7	3.87	382	1	M2
20624	14	2.0	3.59	344	1	M2
19824	14	2.0	3.17	394	1	M2
17412	15	2.1	3.14	376	1	M2
19799	12	1.7	3.08	389	2	M2
19801	11	1.6	2.63	336	1	M2
19798	14	2.0	2.51	419	2	M2
17404	11	1.6	2.49	365	1	M2
21083	1	0.1	0.15	507	4	M3
18438	1	0.1	0.33	496	4	M3
21241	3	0.4	0.57	476	3	M1
21090	3	0.4	0.79	467	4	M3
18437	7	1.0	1.22	453	4	M3
22082	1	0.1	0.22	444	4	M3
20626	2	0.3	0.37	443	3	M1
20631	1	0.1	0.15	437	3	M1
20975	1	0.1	0.33	436	4	M4
21087	1	0.1	0.15	436	4	-
Control						
17403	19	2.7	4.18	361	1	M2
21092	13	1.9	3.03	492	4	*F. stricta*

[a] number of times an accession was grazed during the seven trial days
[b] relative palatability index
[c] *in vitro* dry matter digestibility in the dry season
[d] see Figure 5.3 and Table 5.2
[e] see Table 5.1

Nineteen of the 20 semi-erect accessions were classified as morphotype M4 (light pink flowers in densely congested racemes and strongly pubescent stems and petioles) and form a complex of three agronomic groups (5, 6 and 7) with particularly low DM production and low digestibility in the dry season. The five semi-erect accessions belonging to morphotype M3 (less than 20 dark pink or white flowers in very lax racemes which usually do not exceed the length of the petiole) are clustered together with the remaining M4 accession CIAT 20975, composing the agronomic Group 4 with particularly high digestibility.

The accessions with the highest IVDMD were CIAT 18437, CIAT 18438, CIAT 21083, CIAT 21090, CIAT 21241 and the reference accession *F. stricta* CIAT 21092. The most productive accessions were CIAT 7184, CIAT 21090, CIAT 21241, CIAT 21248, CIAT 21249, CIAT 21519, CIAT 21529, CIAT 21580 and CPI-104890 with a total

DM production of more than 350 g plant^{-1} in the wet and more than 200 g plant^{-1} in the dry season. Accessions CIAT 7184, CIAT 17411, CIAT 19457, CIAT 19800, CIAT 20621, CIAT 20626 and CIAT 20976 had DM yields in the dry season which were more than 70% of those in the wet season.

In the cafeteria trial *F. macrophylla* was, in general, palatable to cattle in the dry season. In the wet season, the animals refused to consume the plants and the trial therefore had to be aborted after five days. In the dry season, the palatability of immature foliage was observed to be considerably higher than that of mature foliage. Of the 69 accessions evaluated, 42 (60%) were consumed in the dry season. Results also showed that 16 accessions were of moderate palatability (RPI > 2.0), but the majority (26 accessions) had very low or low palatability (RPI ranging from 0.1 to 1.8). CIAT 17409, CIAT 20616 and CIAT 17407 and control CIAT 17403 were the most palatable accessions.

The reference accession *F. stricta* (CIAT 21092) was among the ten most palatable accessions. Unexpectedly, a highly significant negative correlation (r = -0.48, $P < 0.01$) was found between IVDMD and palatability. The accessions which were most frequently consumed, had very low to moderate digestibility (Table 5.3). In contrast, accessions CIAT 18437, CIAT 21090 and CIAT 21083 which had the highest digestibility, had low to very low relative palatability indices (1.2, 0.8 and 0.2, respectively).

Discussion

Intra-specific variability of agronomic and forage quality parameters

Agronomic characteristics and forage quality differed greatly among accessions and among morphotypes. This was to be expected, as the study included a wide range of *F. macrophylla* accessions that also differed morphologically, hence presenting great genetic diversity. Large differences existed in DM production and IVDMD, and to a lesser extent in CP content. As expected, DM production was higher in the wet than in the dry season.

Plant height, plant diameter and DM production differed markedly among the four morphotypes (Figure 5.4). The erect *F. macrophylla* accessions (M1) had the largest plant height, diameter and DM production, followed by the semi-erect morphotypes M2 and M3. Potential uses would be forage, living barriers for erosion control, live soil cover or mulch, and firewood. The semi-erect accessions classified as morphotype M4 had the lowest values, and in addition very low regrowth capacity. Accessions belonging to this morphotype would not be recommended for uses such as living barriers or as forage due to their low plant height, poor regrowth capacity and DM production.

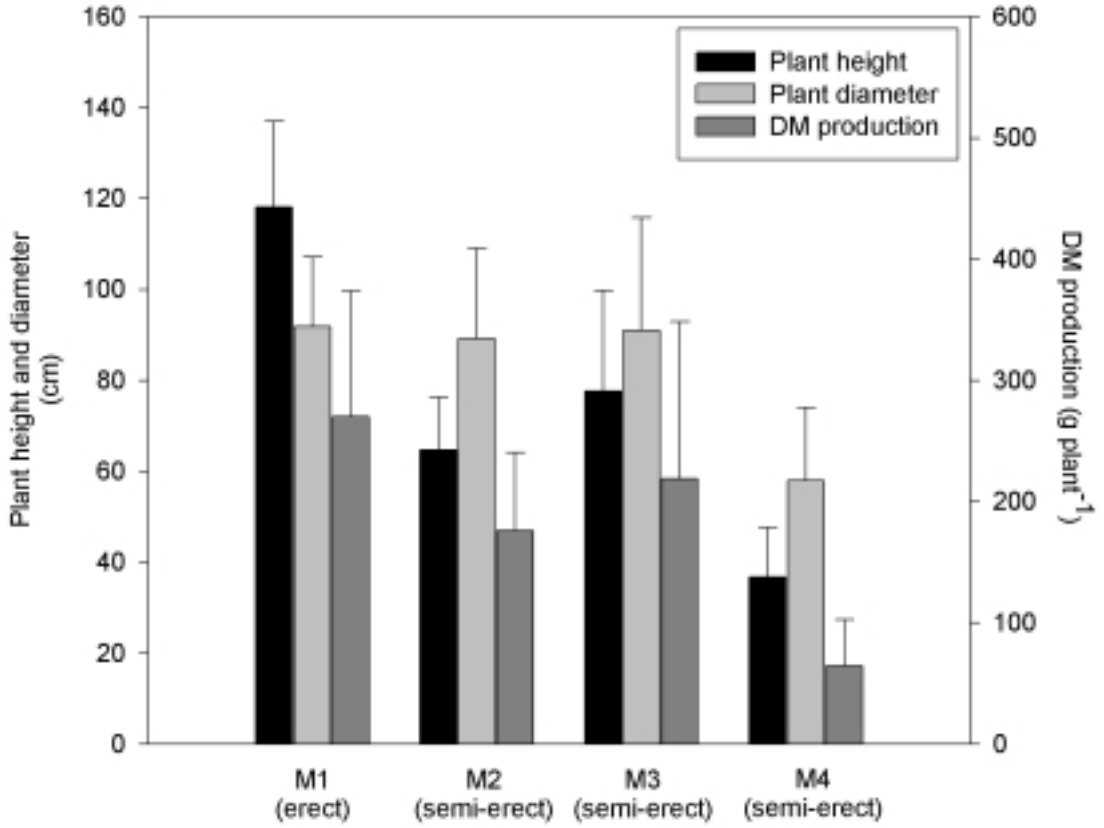

Figure 5.4 Plant height and diameter, dry matter (DM) production and *in vitro* dry matter digestibility (IVDMD) of the four *Flemingia macrophylla* morphotypes (average of two evaluation cuts each during the wet and dry season)

However, they might be useful as live soil cover for weed control due to their horizontal growth. Morphological (Chapter 4) and molecular marker data (Chapter 7) provide evidence that the different morphotypes may represent different taxa. A subset of accessions should be considered for further evaluations, including accessions representing all four morphotypes, as well as the most promising accessions identified in this study.

DM production in the dry season (122 g plant^{-1} ± 75) was on average 57% of that of the wet season, and in the case of the three most promising accessions (CIAT 18437, CIAT 21083 and CIAT 21090), it was 67%, 46% and 39%, respectively. However, in one of the harvest months at the end of a dry season (September 2001), rainfall was up to 100 mm, and therefore the season cannot be classified as the usual "dry" period following the rainy season, but rather as an extension to the period of minimum rainfall. Selected *F. macrophylla* accessions should be tested in drier and monomodal environments in order to better assess their drought tolerance.

Digestibility ranged from 307 to 543 g kg^{-1}, with a maximum of 507 g kg^{-1} in the dry and 543 g kg^{-1} in the wet season. These levels of digestibility are lower than those reported for *Cratylia argentea* (Desv.) O. Kuntze (589 to 690 g kg-1, Andersson *et al.* 2005c, Chapter 2), a shrub legume also well adapted to acid soils, and are in agreement with the findings of other authors, who classify the nutritive quality of *F. macrophylla* as lower than that of *Leucaena leucocephala* (Lam.) de Wit., *Gliricidia sepium* (Jacq.) Walp. and *C. argentea*, but higher than that of *Calliandra calothyrsus* Meisn. (Shelton 2001; Lascano *et al.* 2002).

The results also demonstrated that *F. macrophylla* (and the *F. stricta* reference accession), when offered exclusively, was palatable during the dry season at the experiment site, but was of low palatability in the rainy season, as in agreement with other researchers (Ansah-Adjaye 1977; Asare *et al.* 1984; Schultze-Kraft *et al.* 1989; Kaitho *et al.* 1996; Barnes 1997; Kexian *et al.* 1998). In the tropics, cattle usually prefer green grass (when present) over legumes, particularly in the rainy season (Lascano 1987; Humphreys 1991). In the dry season, however, grass is frequently not available in sufficient quantities.

The negative correlation detected between palatability and IVDMD, and the low consumption of *F. macrophylla* in the wet season could have been due to high concentration of soluble condensed tannins which have been observed to be higher in forage harvested in the wet season as compared to the dry season (Barahona 1999). However, forage quality analysis of a subset of 25 *F. macrophylla* accessions showed that for the majority of the accessions the tannin concentration was higher in the dry than in the wet season (Chapter 6, Andersson *et al.* 2005a). 'Palatability' is not constant because it is influenced by the environment, prior learning, time of adjustment of animals to new feeds, different species and even breeds of animals (Faint *et al.* 1998; Shelton 2000). Also, complex genotype x environment interactions can influence palatability indirectly by affecting forage quality, e.g. via soil and fertility effects and precipitation (Schmidt 2001).

Another factor that could have influenced palatability is morphotype. Morphological attributes and biochemical compounds influence the probability and intensity of grazing by affecting tissue accessibility and palatability (Briske 1991). As mentioned above, the different *F. macrophylla* morphotypes likely represent different taxa, and morphological and chemical differences among them might influence their relative acceptability.

For example, semi-erect accessions belonging to the morphotype M4 have strongly pubescent stems and petioles whereas all other accessions are glabrous or only slightly pubescent. Leaf area also varies between morphotypes, as well as leaf thickness and roughness. Accessions of the morphotypes M2 and M3 have leaves of intermediate size which are relatively thin and soft, whereas leaves of M4 plants are thicker and rougher. *F. stricta* leaves are conspicuously large, and of a very rough, coriaceous texture (Chapter 4). Leaf related morphological

characteristics such as silica bodies, cuticular waxes and pubescence can act as mechanical deterrents directly influencing palatability (Briske 1986). The negative effect of pubescence on palatability, however, is documented only for ants and other small herbivores (Haynes and Gage 1981; Ribeiro *et al.* 1994) but not for cattle.

Chemical composition, which includes antinutritive components such as polyphenols, and leaf toughness are more important determinants of palatability, both for insect herbivores as well as livestock (Feeny 1976; Lowman and Box 1983; Coley and Barone 1996; Foley *et al.* 1999). With respect to chemical characteristics, differences were detected in forage quality from condensed tannin concentration and monomer composition measurements within *Flemingia* species as well as among *F. macrophylla* morphotypes. Particularly, M3 accessions and the *F. stricta* reference accession were virtually free of condensed tannins (Chapter 6).

The majority of the ten most palatable *F. macrophylla* accessions belonged to the same agronomic cluster (Group 1) (Table 5.3). Eighteen of the 19 accessions of that group were palatable, and all were morphotype M2. The only unpalatable accession in this group, CIAT 19457, was morphotype M1. In general, the following pattern was observed: morphotype M2 was most frequently consumed as well as the *F. stricta* reference accession, whereas the other morphotypes were consumed only rarely (Figure 5.5).

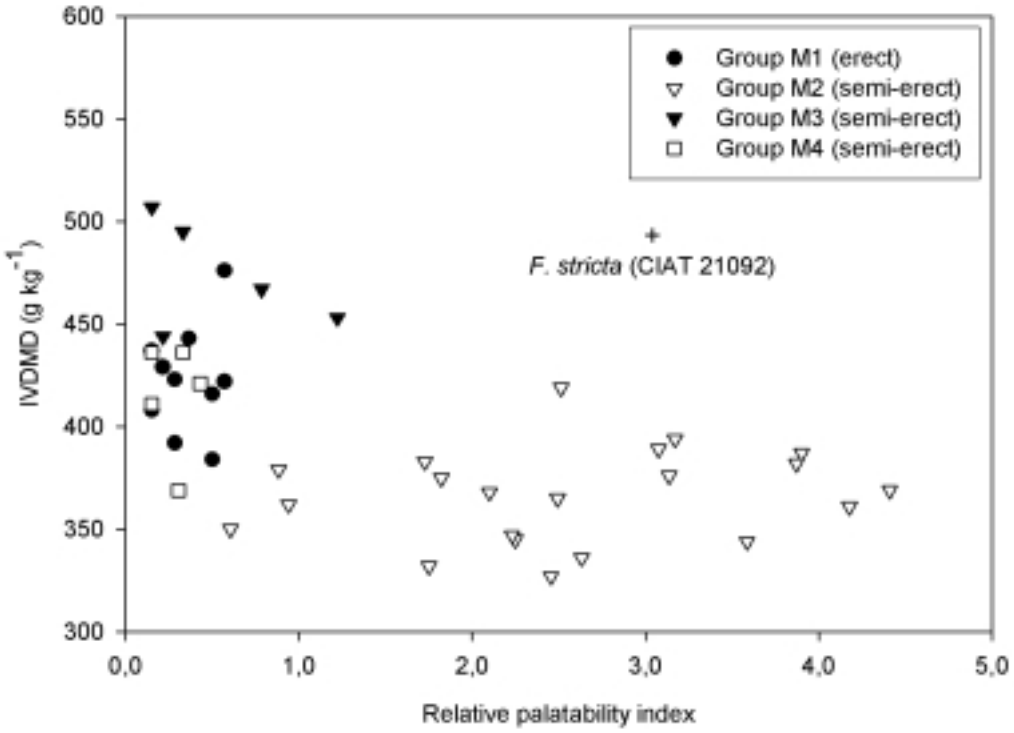

Figure 5.5 Relationship between digestibility and palatability of the four *Flemingia macrophylla* morphotypes and the *F. stricta* reference accession

Table 5.4 Main agronomic and forage quality characteristics of selected *Flemingia macrophylla* genotypes and the control accession CIAT 17403

Accession (CIAT No.)	DM yield (g plant^{-1})		IVDMD (g kg^{-1})		CP (g kg^{-1})		Days to flowering[a,b]	SP (g plant^{-1})[b]
	wet	dry	wet	dry	wet	dry		
18437	239	159	512	453	220	226	303 (-)	0
21083	317	145	495	507	227	183	381 (168)	0
21090	520	201	541	467	226	178	355 (168)	4
17403 (Control)	262	153	415	361	216	211	259 (142)	18

Values are the average from two cuts per season after eight weeks of regrowth.
IVDMD, *in vitro* dry matter digestibility; CP, crude protein; SP, seed production
[a] after planting (after cutting)
[b] see Chapter 4

Further agronomic evaluation should therefore focus on the *F. macrophylla* morphotype M2 and on *F. stricta* on the one hand, due to their acceptability by cattle. On the other hand, more detailed studies are needed to elucidate the relationship between forage quality (digestibility, tannins) and palatability of different *F. macrophylla* morphotypes, particularly of those with high digestibility (morphotypes M3 and M1).

Identification of promising accessions

In general, the dry matter production and forage quality (particularly *in vitro* dry matter digestibility) of *F. macrophylla* are lower than for other tropical forage shrubs or trees such as *L. leucocephala* or *G. sepium*. The particular niche of *F. macrophylla* is on acid, low-fertility soils, where it clearly outcompetes these species in terms of DM production (Schultze-Kraft 1996). Low IVDMD and palatability, however, were a limiting factor for its use as forage for cattle. The results of this study show, that there is scope for plant improvement in terms of higher IVDMD (up to 543 g kg^{-1}), but palatability of the accessions with highest IVDMD was low.

The semi-erect accessions CIAT 18437, CIAT 21083 and CIAT 21090 (all morphotype M3) were selected as promising for further evaluation because they combined high digestibility in the dry and wet seasons with high DM production (Table 5.4). These accessions had higher DM production and IVDMD than the control CIAT 17403. However, they were only moderately palatable when compared to other forage legumes such as *L. leucocephala* or *C. argentea* (Shelton 2001; Lascano *et al.* 2002).

Palatability should be more thoroughly assessed in cafeteria trials with a selected set of accessions at different stages of maturity, and also with cut material offered fresh, wilted, and as silage or hay, including adequate adaptation periods for livestock. Offering a mixture of a palatable forage species with high-quality accessions of *F. macrophylla* is another option to overcome these limitations, while at the same time increasing protein availability and animal

live weight gains (Barry and McNabb 1999; Fausto-Lanting 1999). Other uses of *F. macrophylla* include its utilization as erosion barrier and mulch for soil conservation.

Seed production of the promising accessions at the experimental site was low (on average less than 5 g plant^{-1}, Chapter 4), and this may limit their dissemination as forage plants. Therefore particular attention needs to be paid also to the enhancement of seed production of these accessions, e.g. through evaluations in different environments (e.g. latitudes, altitudes) and micronutrient input. Preliminary tests with stem cuttings showed that vegetative propagation is also feasible, but this might negatively affect the species' drought tolerance because root development of cuttings may be shallow and devoid of a strong taproot, as frequently observed in other woody species (Kretschmer Jr. 1978; Hartmann *et al.* 2002).

Accession CIAT 18437 was lost in the morphology trial after cutting in the period of minimum rainfall. However, in the agronomy trial all plants of this accession showed excellent vigour and regrowth capacity under the two-year regime of frequent cutting (every two weeks). It is therefore assumed that the adaptation problem in the morphology trial was due to chance or unfavourable soil conditions at this specific micro-site.

Conclusions

F. macrophylla offers great biomass potential, particularly on acid, low-fertility soils. Furthermore, the results of this study demonstrate that there is scope for plant improvement in terms of forage quality (*in vitro* dry matter digestibility), which is currently the limiting factor for its use as forage. The low palatability observed in the preliminary grazing trial and the negative correlation with IVDMD at present seem to present a restriction, but may be overcome by forage treatment (e.g. cut, wilted, or as hay), longer time of adjustment of cattle to the new feed, or by offering mixtures with more palatable species. Further studies are needed to test these possibilities. Moreover, *F. macrophylla* can be used for multiple purposes, such as living fences, erosion barrier, and for green manure and mulching.

Chapter 6

Forage quality and tannin concentration and composition of a collection of the tropical shrub legume *Flemingia macrophylla*

Abstract

A collection of 23 *Flemingia macrophylla* (Willd.) Merrill accessions of different growth habits and contrasting digestibility and one *F. stricta* reference accession were assessed for forage quality with particular emphasis on digestibility, condensed tannin concentration and fiber content. Large differences in *in vitro* dry matter digestibility (IVDMD; 356 to 598 g kg^{-1}), content of crude protein (CP) (121 to 254 g kg^{-1}) and extractable condensed tannins (CT; 0 to 268 g kg^{-1}), protein-binding capacity of extractable CT or astringency (1.7 to 7.9 protein-binding entities, PBE) and monomer composition of extractable CT were detected. IVDMD and extractable CT were negatively correlated, and extractable CT was positively correlated with protein-binding capacity. Prodelphinidin was positively, and propelargonidin negatively correlated with protein-binding capacity of extractable CT. The accessions CIAT 18438, CIAT 21083, CIAT 21090 and CIAT 22082 were superior to the most widely used accession CIAT 17403 in terms of forage quality and could be an option in production systems with acid, infertile soils. In future evaluations, particular attention needs to be paid to chemical and structural features related to the composition of extractable condensed tannins and their effect on nitrogen utilization by ruminants.

Keywords: condensed tannins; digestibility; *Flemingia macrophylla*; monomer composition; forage quality; polyphenols; protein-binding capacity; tannin astringency; tropical legume

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Introduction

Flemingia macrophylla (Willd.) Merrill (syn. *F. congesta, Moghania macrophylla*) is a drought-tolerant, perennial, multipurpose shrub legume especially suited to low-input smallholder production systems in the sub-humid and humid tropics (Andersson *et al.* 2002).

One of the limitations of the species is the presence of anti-nutritive components, in particular polyphenols, combined with low palatability to cattle (Thomas and Schultze-Kraft 1990; Jackson *et al.* 1996; Barahona *et al.* 1997). Its particular advantages in comparison with other drought-tolerant legume shrubs adapted to infertile soils but free of tannins such as *Cratylia argentea* (Desv.) O. Kuntze (Lascano *et al.* 2002), are its adaptation to higher altitudes (up to 2000 m above sea level) and its wide range of uses such as soil improving cover crop or mulch (Budelman 1988), erosion barrier hedge (Budelman and Siregar 1997), firewood and shade-providing shrub in young coffee and cocoa plantations (Banful *et al.* 2000). When intercropped with cassava and maize, it significantly increased the yields of the associated crops (Hauser *et al.* 2000; Tien Dung *et al.* 2005). Therefore, research on *F. macrophylla* is particularly important due to its limited forage quality, and special attention should be paid to the identification of accessions with low tannin content.

A morphological characterization of the available world collection of 69 accessions identified four morphotypes (M) differing mainly in growth, flower and seed characteristics, and in an agronomic evaluation, large variability was detected among accessions as well as among morphotypes (Chapter 4 and 5, Andersson *et al.* 2005b). Variability was particularly large in terms of agronomic traits (dry matter production, plant height, regrowth capacity), forage quality (*in vitro* dry matter digestibility, crude protein content) and palatability. Out of 69 accessions evaluated, 42 (60%) were consumed by cattle in the period of minimum rainfall. The semi-erect morphotype M2 was most frequently consumed as well as the *F. stricta* reference accession, whereas the other morphotypes (erect M1 and semi-erect M3 and M4) were consumed only rarely. Unexpectedly, a highly significant negative correlation was found between *in vitro* digestibility and palatability. It was hypothesised that morphological (e.g. leaf toughness) and chemical (e.g. polyphenols) differences related to morphotype might have affected palatability.

The present research complements previous studies by analysing in more detail the forage quality of a subset of 23 *F. macrophylla* accessions of contrasting digestibility, along with one *F. stricta* accession included as reference. *In vitro* dry matter digestibility, crude protein and fibre (NDF, ADF, N-ADF) content were determined, together with tannin content and composition.

Materials and methods

Selection of the accessions

Twenty-three *Flemingia macrophylla* accessions, representative of the world collection of 69 accessions in terms of forage quality, were selected based on *in vitro* dry matter digestibility, crude protein content and morphotype, according to results from an agronomic evaluation (Chapter 5**;** Table 6.1). The morphotypes (M) were: M1 (*n* = 8): erect accessions with stalked racemes, numerous light pink flowers and black seeds; M2 (*n* = 3): semi-erect accessions with dark pink flowers in densely congested racemes and glabrous or slightly pubescent stems and petioles; M3 (*n* = 5): semi-erect accessions with less than 20 dark pink or white flowers in very lax racemes which usually do not exceed the length of the petiole; and M4 (*n* = 5): semi-erect accessions with light pink flowers in densely congested racemes and strongly pubescent stems and petioles (Chapter 4); as well as one accession (CIAT 21087) with undetermined morphotype (due to insufficient morphological data).

The selected subset included five high, ten intermediate and seven low-digestibility accessions belonging to four different morphotypes. Accession CIAT 17403 (morphotype M2) was included as control because it had frequently been used in other experiments (Thomas and Schultze-Kraft 1990; Cano *et al.* 1994). CIAT 21092 (an accession of *Flemingia stricta* Roxb.) was included as an additional reference accession due to its vigour and remarkably big leaves, and its relatively high palatability to cattle (Chapter 5).

Evaluation site

The experiment reported here was carried out in 2002 with plants grown at the Quilichao Experimental Station of the International Centre for Tropical Agriculture (CIAT) in the Cauca department, Colombia (03°06' N, 76°31' W; 990 m elevation, average annual temperature 23 °C, average annual rainfall 1800 mm; actual rainfall in 2001 and 2002, 1345 and 1265 mm respectively). The soil of the experimental plots is an acid Ultisol (pH = 5.3) with 76% Al saturation, medium P content (6 ppm Bray II) and high organic matter content (7.4%).

The seeds were sown into Jiffy pots in the greenhouse and inoculated with *Bradyrhizobium* strain CIAT 4099. After six weeks, the seedlings were transplanted to the field in 9-plant single-row plots, at a distance of 1 m between plants and 1.5 m between rows. The experimental design was a Randomised Complete Block with three replications. Two weeks after transplanting individual plants were fertilized at a rate of 40, 50, 20 and 0.5 kg ha^{-1} of P, K, Mg and Mo, respectively.

Table 6.1 Geographic origin of 23 *Flemingia macrophylla* and one *F. stricta* accession.

CIAT No.	Country of origin[1]	State/Province/ Region	Latitude	Longitude	Altitude (m asl)	Rainfall (mm)	Morpho-type[2]
17407	THA	Southern Thailand	08° 18' N	99° 55' E	50	2410	M2
18437	IDN	West Sumatra	00° 29' S	100° 52' E	190	2430	M2
18438	THA	Eastern Thailand	12° 47' N	101° 44' E	40	2390	M3
19457	PNG	Papua New Guinea	06° 02' S	145° 22' E	1630	1930	M1
20616	IDN	Aceh, Sumatra	04° 44' N	96° 45' E	690	1730	M2
20621	IDN	Aceh, Sumatra	03° 41' N	97° 37' E	1110	2390	M2
20622	IDN	North Sumatra	02° 34' N	98° 32' E	1350	2330	M1
20744	COL	Tolima	03° 43' N	75° 32' W	930	2890	M1
20975	CHN	Hainan	18° 49' N	109° 17' E	230	1600	M4
20976	CHN	Hainan	18° 45' N	109° 30' E	330	1800	M4
21083	THA	Northern Thailand	18° 40' N	97° 56' E	620	1300	M3
21087	THA	Northern Thailand	18° 51' N	98° 52' E	700	1250	-
21090	THA	Northern Thailand	19° 12' N	99° 31' E	550	1350	M3
21241	COL	Meta	03° 32' N	73° 46' W	400	2920	M1
21249	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	M1
21529	IDN	Java	07° 45' S	110° 20' E	750	1500	M1
21580	CMR	Centre	03° 52' N	11° 31' E	760	1680	M1
21982	VNM	North Central Coast	16° 24' N	107° 32' E	70	2900	M4
21990	VNM	South Central Coast	15° 28' N	108° 22' E	80	2210	M4
21992	VNM	South Central Coast	14° 51' N	108° 52' E	40	2170	M4
22082	THA	Northeastern Thailand	18° 14' N	103° 10' E	190	2230	M3
J-001	COL	Meta	03° 13' N	73° 15' W	300	n.a.	M1
Reference accessions:							
17403	THA	Southern Thailand	10° 04' N	99° 04' E	40	1870	M2
21092	THA	Northern Thailand	20° 26' N	99° 54' E	500	1570	*F. stricta*

[1] CHN, China; CMR, Cameroon; COL, Colombia; IDN, Indonesia; PNG, Papua New Guinea; THA, Thailand; VNM, Vietnam.

[2] Morphotypes according to **Chapter 4**: M1, erect accessions (stalked racemes with numerous light pink flowers and black seeds); M2, semi-erect accessions with dark pink flowers in densely congested racemes and glabrous or slightly pubescent stems and petioles; M3, semi-erect accessions with less than 20 dark pink or white flowers in very lax racemes, which usually do not exceed the length of the petiole; M4, semi-erect accessions with light pink flowers in densely congested racemes and strongly pubescent stems and petioles. For accession CIAT 21087 the morphotype could not be determined due to missing morphological data.

Forage quality analysis

During one wet (April 2002) and one dry season (August 2002), fully expanded young leaves of 8-week regrowth were harvested from two year old plants, freeze-dried and ground to pass a 1-mm screen in a Wiley mill. *In vitro* dry matter digestibility (IVDMD) was determined using the two-stage technique (Tilley and Terry 1963) modified by Harris (1970). Total nitrogen (N) was determined by the Kjeldahl procedure (AOAC, 2003) and multiplied by 6.25 to obtain an estimate of crude protein (CP). For determination of neutral and acid detergent fibre (NDF, ADF) and acid detergent fibre-bound nitrogen (N-ADF) the method of Soest *et al.* (1991) was used. Sodium sulphite was added to the neutral detergent solution to partially remove tannin-protein complexes (Robbins *et al.* 1987).

Extractable and fibre-bound condensed tannins (CT) were determined by the procedure suggested by Terrill *et al.* (1992) as modified by Barahona *et al.* (2003). A standard curve was obtained using a mixture of tannins extracted from nine randomly chosen *F. macrophylla* accessions (CIAT 17403, CIAT 18437, CIAT 20621, CIAT 21083, CIAT 21241, CIAT 21580, CIAT 21990, CIAT 22082, CIAT 22327) and purified on a column packed with Sephadex LH-20 (GE Healthcare Bio-Sciences Corp., NY, USA) (Terrill *et al.* 1992). Total CT (TCT) content was obtained by summing extractable and bound CT, and tannin extractability as extractable CT/TCT. Given that a mixture of nine accessions was used to obtain a single standard curve it is acknowledged that this could have affected the accuracy of the results of extractable and bound CT concentration.

For determination of the protein-binding capacity (astringency) of extractable CT the radial diffusion technique of Hagerman (1987) as modified by Lareo *et al.* (1990) was used. Protein-binding capacity (PBC) of the extractable CT was expressed as protein-binding entities (PBE), calculated as: PBE = volume of the agarose cylinder ring x protein concentration, with ring volume = π x h x (r_1^2 - r_2^2); r_1 = outer ring radius; r_2 = inner ring radius and h = well height. The monomer composition of the extractable CT fraction was determined by high-performance liquid chromatography (HPLC) (Shimadzu CL 10A, Shimadzu Inc., Canby, USA) (Barahona 1999). A 1-ml aliquot of extractable CT was evaporated and the residue dissolved in HCl-methanol (1:99, v/v). Ten-µl aliquots were injected into an 18 x 10 cm Nova Pack C18 (Waters Corp., Milford, USA) column. Aqueous acetic acid (95:5 v/v, solvent A) and methanol (solvent B) were used for gradient elution at 2 ml min^{-1}. The gradient profile was 60 - 30% A (0.02 - 4.30 min) and 35 - 60% B (4.30 - 8.00 min). Total flow time was 8 min and absorbance was measured at 520 nm with an SPD 10AV UV-visible detector (Shimadzu Inc., Canby, USA). Delphinidin, cyanidin and pelargonidin standards from APIN CHEM (Oxon, UK) were used for peak identification.

Laboratory determination of IVDMD, CP and fibre content as well as of monomer composition of the extractable CT fraction was carried out in duplicate, while the determination of extractable and bound CT and protein-binding capacity was carried out in triplicate. If the standard deviation (SD) among laboratory replicates exceeded 2.5 g kg^{-1} (IVDMD, CP and fibre content), 4.0 g kg^{-1} (extractable CT), 2.0 g kg^{-1} (bound CT) or 5.0 PBE (protein-binding capacity), then measurements were repeated. Results of the analysis of CT monomer composition with HPLC were extremely variable among field replications as well as between laboratory duplicate samples and seasons. As this is the first study of monomer composition of more than one *F. macrophylla* accession, only the results of those seven accessions are presented where the standard deviation SD among field repetitions, laboratory replicates and seasons did not exceed 10%.

Statistical analysis

The experimental design was a Randomised Complete Block with 25 accessions (treatments) and three replications per season. Correlations were calculated between forage quality traits using Pearson's Correlation Coefficient. Data were subjected to analysis of variance (GLM-ANOVA procedure) using a model containing seasons, replications (blocks), accessions and experimental error as the main sources of variation. Laboratory duplicate or triplicate samples were averaged for each experimental unit prior to the analysis of variance so that a pooled estimate of sampling variance was not obtained. Means were separated at $P < 0.01$ using the Ryan-Einot-Gabriel-Welsch Multiple Range test. All analyses were performed using the SAS program package version 8.2 (SAS Institute Inc. 1999).

Results

The chemical composition of *F. macrophylla* differed greatly among accessions. Digestibility ranged from 356 to 598 g kg^{-1} and CP content from 121 to 254 g kg^{-1} (Table 6.2). NDF ranged from 281 to 425 g kg^{-1} and ADF from 176 to 302 g kg^{-1}. The unavailable nitrogen fraction associated to the cell wall (N-ADF) ranged from 50 to 185 g kg^{-1}. Condensed tannins showed great variability in both concentration and composition. Total CT (TCT) content ranged from 12 to 291 g kg^{-1}, extractable CT from 0 to 268 g kg^{-1} and bound CT from 3 to 41 g kg^{-1}. Protein-binding capacity of the extractable CT ranged from 1.7 to 7.9 protein-binding entities (PBE). Digestibility (IVDMD) was negatively correlated with sCT (r = -0.63, $P < 0.01$) and to a lesser extent with ADF (r = -0.44, $P < 0.01$) for both seasons.

Correlation coefficients for the traits digestibility, fiber and condensed tannin content and protein-binding capacity are shown in Table 6.3. Genotypes differed ($P < 0.01$) for each of the forage quality traits and genotype x season interaction was detected for IVDMD, extractable CT and bound CT, and PBC. Fifteen of the 23 *F. macrophylla* and the *F. stricta* reference accessions were stable across seasons for all traits, whereas eight accessions showed interactions (Table 6.4 and Table 6.5).

Accessions CIAT 18438 and CIAT 21087 had significantly lower (LSD 35.04, $P < 0.05$) digestibility in the dry than in the wet season. Accessions CIAT 20616, CIAT 21990 and J-001 had significantly higher (LSD 35.06, $P < 0.05$) extractable CT content and CIAT 21241 and CIAT 21992 significantly higher (LSD 0.96, $P < 0.05$) protein-binding capacity in the dry than in the wet season. The bound CT content of CIAT 20622 was significantly lower (LSD 8.82, $P < 0.05$) in the dry than in the wet season. In general, however, this had little agronomic implications as only accessions with a generally high forage quality across seasons are of interest for further evaluation.

Table 6.2 Means and ranges of forage quality traits of 23 *Flemingia macrophylla* and one *F. stricta* accessions with contrasting digestibility and crude protein content in wet and dry season

Forage quality trait[a]	Season	Mean	Range	SEM
IVDMD (g kg^{-1} DM)	wet	466	369 – 598	6.3
	dry	433	356 – 525	4.6
CP (g kg^{-1} DM)	wet	213	127 – 254	2.4
	dry	205	121 – 229	2.3
NDF (g kg^{-1} DM)	wet	344	281 – 412	4.0
	dry	357	307 – 425	3.7
ADF (g kg^{-1} DM)	wet	229	176 – 300	2.7
	dry	245	213 – 302	2.8
N-ADF (g kg^{-1} DM)	wet	115	80 –177	2.6
	dry	114	50 – 185	2.9
extractable CT (g kg^{-1} DM)	wet	75	0 – 191	5.4
	dry	97	0 – 268	7.2
fibre-bound CT (g kg^{-1} DM)	wet	20	3 – 39	0.8
	dry	21	0 – 41	0.9
TCT (extractable + bound CT)	wet	95	12 – 214	5.7
	dry	118	0 – 291	7.1
PBC (PBE)	wet	4.7	1.7 – 7.7	0.17
	dry	5.3	2.2 – 7.9	0.19

[a] Data of one evaluation cut per season after eight weeks of regrowth.
Abbreviations: IVDMD, *in vitro* dry matter digestibility; CP, crude protein; NDF, neutral detergent fibre; ADF, acid detergent fibre; N-ADF, nitrogen bound to acid detergent fibre; CT, condensed tannins; TCT, total condensed tannins; PBC, protein-binding capacity; PBE, protein-binding entities

Table 6.3 Correlation coefficients (*r*) of 23 *Flemingia macrophylla* and one *F. stricta* accessions assessed for digestibility, fibre and condensed tannin content and protein-binding capacity ($P < 0.01$)

	NDF	ADF	N-ADF	extractable CT	fibre-bound CT	PBC
IVDMD	-0.255	-0.377	ns	-0.626	ns	-0.613
NDF	–	0.589	0.337	ns	ns	ns
ADF		–	ns	ns	ns	ns
N-ADF			–	ns	ns	ns
extractable CT				–	ns	0.698
fibre-bound CT					–	ns

IVDMD, *in vitro* dry matter digestibility; NDF, neutral detergent fibre; ADF, acid detergent fibre; N-ADF, nitrogen bound to acid detergent fibre; CT, tannins; PBC, protein-binding capacity; ns, not significant

Table 6.4 *Flemingia macrophylla* accessions stable across seasons for the forage quality traits *in vitro* dry matter digestibility (IVDMD), extractable and fibre-bound condensed tannin (CT) content and protein-binding capacity (PBC)

Accession (CIAT No.)	IVDMD (g kg^{-1})	sCT (g kg^{-1})	bCT (g kg^{-1})	PBC (PBE)[a]
17407	423.6	93.8	21.9	5.4
18437	492.1	59.0	22.8	4.4
19457	417.6	96.7	23.2	6.3
20744	397.0	156.1	17.5	5.7
20975	463.1	129.1	20.7	5.2
20976	470.8	119.5	16.2	5.1
20621	433.6	150.6	18.3	5.7
21083	501.6	0.5	16.7	2.1
21090	534.8	ND	19.9	2.3
21249	432.8	98.7	24.9	5.1
21529	431.8	93.8	27.4	5.1
21580	429.2	75.5	21.8	5.3
21982	447.8	102.7	22.0	6.9
22082	473.9	2.5	19.1	2.8
18438	interaction	8.2	18.9	3.6
20616	448.1	interaction	22.1	6.6
20622	422.0	105.1	interaction	5.4
21087	interaction	68.9	18.0	5.4
21241	412.9	90.2	23.4	interaction
21990	383.3	interaction	26.4	6.7
21992	447.0	99.2	22.8	interaction
J-001	446.6	interaction	21.4	4.9
17403 (Control)	430.9	69.7	22.8	4.8
21092 (*F. stricta*)	477.5	52.0	18.7	3.2
LSD ($P < 0.05$)	27.15	22.76	6.82	0.58

[a] PBE, protein-binding entities; ND, not detectable

Table 6.5 *Flemingia macrophylla* accessions showing genotype x season interaction for the forage quality traits *in vitro* dry matter digestibility (IVDMD), extractable and fibre-bound condensed tannin (CT) content and protein-binding capacity (PBC)

Accession (CIAT No.)	IVDMD (g kg^{-1})	sCT (g kg^{-1})	bCT (g kg^{-1})	PBC (PBE)[a]
	wet / dry	wet / dry	wet / dry	wet / dry
18438	562.0 / 473.1	ns	ns	ns
20616	ns	58.7 / 151.5	ns	ns
20622	ns	ns	29.4 / 12.0	ns
21087	539.8 / 444.5	115.2 / 204.0	ns	ns
21241	ns	ns	ns	3.8 / 6.1
21990	ns	ns	ns	ns
21992	ns	ns	ns	5.1 / 7.9
J-001	ns	69.6 / 151.0	ns	ns
LSD ($P < 0.05$)	35.04	35.06	8.82	0.96

[a] PBE, protein-binding entities; wet, period of maximum rainfall; dry, period of minimum rainfall; ns, not significant

Four semi-erect accessions (CIAT 18438, CIAT 21083, CIAT 21090 and CIAT 22082) belonging to the morphotype M3 had very low extractable CT content (< 10 g kg^{-1}) whereas the content of extractable CT of all other *F. macrophylla* accessions and of the *F. stricta* accession (CIAT 21092) ranged from 40 to 194 g kg^{-1}. Also, tannin extractability (extractable CT/TCT) of these four accessions was considerably lower (< 16%) than that of the other accessions (extractability 60-95%), reflecting the fact that the content of bound CT in these accessions was generally not lower than in the rest of the collection. The *F. macrophylla* control as well as the *F. stricta* accession, were intermediate in terms of forage quality, with average IVDMD of 431 and 478 g kg^{-1}, extractable CT contents of 70 and 52 g kg^{-1}, and PBC values (PBE) of 4.8 and 3.2, respectively.

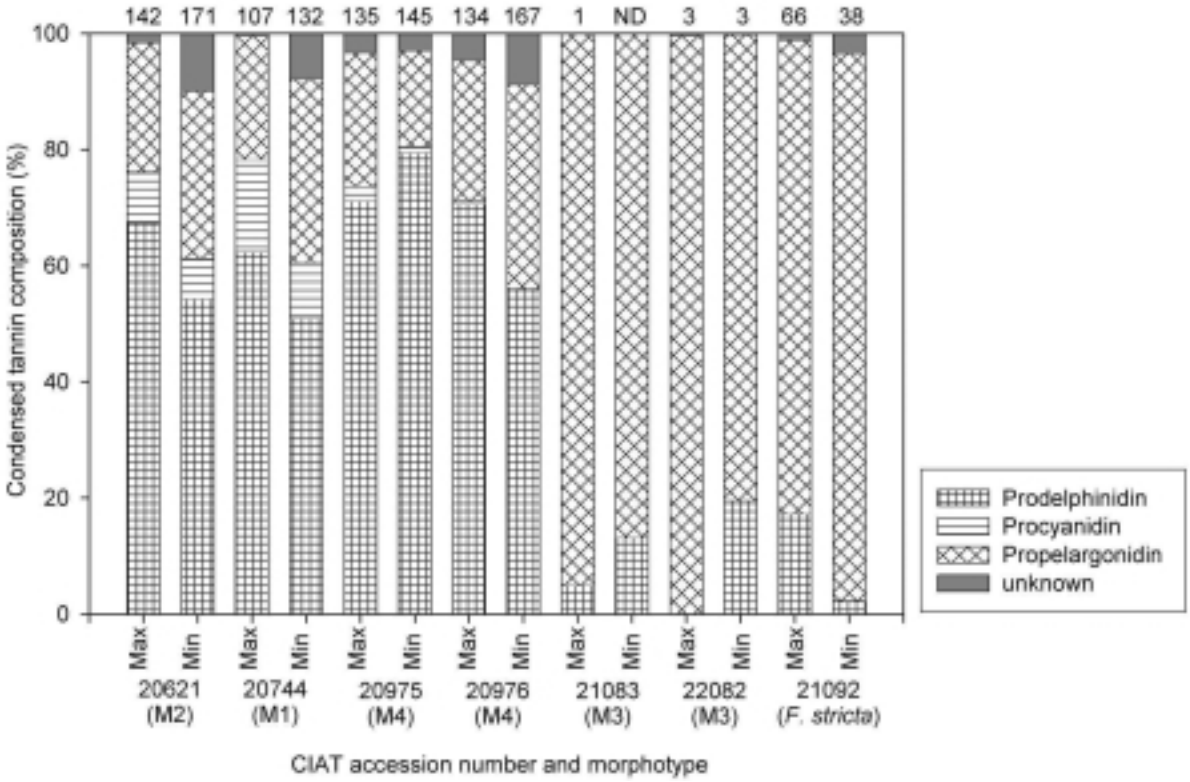

Figure 6.1 Monomer composition (procyanidin: prodelphinidin: propelargonidin ratio) of the extractable condensed tannin (CT) fraction of six *Flemingia macrophylla* and one *F. stricta* accessions in the wet and dry season. M1 to M4, morphotypes 1 to 4 (see text). Extractable CT contents determined by the Butanol-HCl assay are given at the top of the graphic; ND, not detectable.

Differences were detected in the monomer composition of extractable CT of six *F. macrophylla* accessions and the *F. stricta* accession (Figure 6.1). Two *F. macrophylla* accessions (CIAT 21083 and CIAT 22082, both belonging to the group of low extractable CT content identified above) and *F. stricta* were strikingly different from the other four accessions. Their dominant constituent was propelargonidin which ranged from 80 to 100% of total extractable CT, whereas prodelphinidin was less than 20% and procyanidin totally absent. In the other four *F. macrophylla* accessions CIAT 20621, CIAT 20744, CIAT 20975 and CIAT 20976, prodelphinidin made up more than half of the extractable CT (49 to 79%) while propelargonidin ranged from 16 to 38% and procyanidin from 0 to 16%. Propelargonidin was negatively correlated with protein-binding capacity of the extractable CT (r = -0.84, $P < 0.01$) and positively with IVDMD (r = 0.44, $P < 0.01$) for both seasons. In contrast, prodelphinidin was positively correlated with protein-binding capacity of extractable CT (r = 0.84, $P < 0.01$). No difference was detected in extractable CT composition between seasons.

Table 6.6 Forage quality of two selected promising *Flemingia macrophylla* accessions and control CIAT 17403

	Season	CIAT 21083	CIAT 21090	CIAT 17403 (Control)
IVDMD (g kg^{-1} DM)	wet	522.6	557.6	460.7
	dry	480.5	512.0	401.0
CP (g kg^{-1} DM)	wet	244.0	237.1	205.2
	dry	225.0	221.0	200.6
NDF (g kg^{-1} DM)	wet	312.6	329.5	350.6
	dry	332.9	329.3	378.1
ADF (g kg^{-1} DM)	wet	214.4	207.4	203.6
	dry	238.3	232.6	240.9
N-ADF (g kg^{-1} DM)	wet	109.0	119.0	93.0
	dry	110.9	130.3	120.3
extractable CT (g kg^{-1} DM)	wet	1.1	ND	43.2
	dry	0.0	ND	96.2
fibre-bound CT (g kg^{-1} DM)	wet	13.6	15.7	21.2
	dry	18.1	19.9	24.3
PBC (PBE)	wet	1.7	2.0	4.5
	dry	2.4	2.5	5.0
Relative Palatability Index[a] (RPI)	dry	0.15	0.79	4.18
DM production (g plant^{-1})[a]	wet	317	520	262
	dry	145	201	153
Seed production (g plant^{-1})[b]	dry	< 5	< 5	18

Data of one evaluation cut per season after eight weeks of regrowth. IVDMD, *in vitro* dry matter digestibility; CP, crude protein; sCT, soluble condensed tannins; bCT, bound condensed tannins; PBC, protein-binding capacity; PBE, protein-binding entities; DM, dry matter; ND, not detectable

[a] Data from an agronomic evaluation (**Chapter 5**)

[b] Data from a morphological characterization (**Chapter 4**)

The two semi-erect *F. macrophylla* accessions CIAT 21083 and CIAT 21090, identified in Chapter 5 as promising in terms of IVDMD and dry matter (DM) production, were superior to the control CIAT 17403 also in terms of having lower fibre and tannin content, and tannins with lower protein-binding capacity (Table 6.6). Both accessions were virtually free of extractable condensed tannins, and the protein-binding capacity of their tannins was considerably lower than that of CIAT 17403. The seed production of these accessions at the experimental site, however, had been low (on average less than 5 g plant^{-1}), as well as their palatability in a preliminary grazing trial (Table 6.6).

Discussion

Average DM digestibility (453 g kg^{-1}) and CP content (208 g kg^{-1}) across accessions and seasons were in agreement with the variability in the world collection (Chapter 5, Andersson *et al.* 2005c), and higher than recorded for *F. macrophylla* by other authors (Thomas and Schultze-Kraft 1990; Lowry *et al.* 1992; Dzowela *et al.* 1995; Fässler and Lascano 1995; Lascano *et al.* 1995; Barahona *et al.* 2003). The average fibre fractions (ADF and NDF) were lower and N-ADF values higher than those reported by Fässler and Lascano (1995) for the control accession grown at the same location (using the same analytical method). However, the content of extractable CT (91 g kg^{-1}) and bound CT (21 g kg^{-1}) and tannin extractability (69%) were consistent with results reported for *F. macrophylla* by Fässler and Lascano (1995) and Barahona *et al.* (1997; 2003) but lower than measured by Cano *et al.* (1994), Longland *et al.* (1995) and Lascano *et al.* (1995) who used the same analytical method.

Other studies on chemical composition of *Flemingia macrophylla* are scarce and do not permit direct comparisons due to differences in the methodology used to measure tannins. For example, the condensed tannin concentrations of 12 *F. macrophylla* accessions were documented by Jackson *et al.* (1996), and four of these accessions (CIAT 17407, CIAT 21090, CIAT 21092, CIAT 21249) were also included in this study. Results from both studies confirmed that tannin content and extractability differed greatly among accessions, although Jackson *et al.* (1996) used tannins extracted from *Lotus pedunculatus* as standard whereas in the present study a mixture of tannins extracted from nine *F. macrophylla* accessions was used. Furthermore, the ranking of the accessions used in both studies was similar, with accession CIAT 21249 showing the highest extractable CT content and CIAT 21090 the lowest.

It is widely accepted that the extractable condensed tannin fraction depresses digestibility of dry matter, fibre and protein (Rittner and Reed 1992; Norton 2000). The negative correlation of IVDMD with extractable CT and protein-binding capacity confirms these results and corroborates similar findings reported by Lascano *et al.* (1995) for *F. macrophylla* and a

range of other tropical legumes, and by Dalzell *et al.* (1998) and McNeill *et al.* (1998) for *Leucaena* species.

The low reproducibility observed in the analysis of CT monomer composition with HPLC might have been caused by sample treatment. Due to the relatively large number of samples, it was not possible to continue immediately with HPLC analysis after the Butanol-HCl assay. Therefore, after determination of the extractable CT content, samples were evaporated and the residue stored at 4 °C for three days. Only then, were samples were re-dissolved for continuing with the HPLC analysis. It could be possible that refrigeration has affected the composition of condensed tannins. To avoid these problems, in the future samples should be processed immediately, or purified condensed tannins should be used instead of the tannin extract from Butanol-HCl analysis.

The extractable CT fraction of accessions CIAT 20621, CIAT 20744, CIAT 20975 and CIAT 20976 was mostly formed by prodelphinidin and procyanidin (> 60%), whereas propelargonidin (> 80%) was dominant in accessions CIAT 21083 and CIAT 22082 and in *F. stricta*. Although these seven accessions are not representative of the entire collection in terms of forage quality, their monomer composition indicates considerable variation. Jones *et al.* (1976) associated the prodelphinidin content with higher protein-binding capacity of extractable CT, and the nutritional implications of the variation in monomer composition in legumes have also been discussed by Koupai-Abyazani *et al.* (1989), Foo *et al.* (1997), Aerts *et al.* (1999) and Hedqvist *et al.* (2000). Stewart *et al.* (2000) reported major differences in tannin monomer composition between two *Calliandra calothyrsus* provenances: the Patulul provenance contained mainly procyanidin, whereas San Ramón was composed largely of prodelphinidin subunits. Recently, Lascano *et al.* (2003) presented evidence suggesting that the monomer composition of extractable CT in tropical legumes can affect N utilization by ruminants.

It was notable that the two *F. macrophylla* accessions containing mainly propelargonidin (CIAT 21083, CIAT 22082) also showed very low levels of extractable CT in the butanol-HCl assay. The color produced in the butanol-HCl reaction, by which all CT values were estimated, results from the oxidative cleavage of the CT polymers into oligomer flavanol units and their subsequent conversion into colored anthocyanidins (e.g. procyanidin, prodelphinidin, propelargonidin)(Porter *et al.* 1986). Each of the anthocyanidins gives a different color reaction. It thus might be possible that the apparent low values of extractable CT are artifacts caused by low color development from propelargonidin, relative to the other monomers. This would also explain why the PBC values for these 'low CT' accessions are not correspondingly as low, as would be expected.

The results show major differences in tannin concentration and composition between the two *Flemingia* species, among different *F. macrophylla* accessions, but not between seasons. The strong correlations of prodelphinidin (positively correlated) and propelargonidin (negatively correlated) with tannin protein-binding capacity corroborate the findings of Jones *et al.* (1976) and point to a relationship between monomer composition and protein availability in legumes with tannins.

It must be acknowledged, however, that the HPLC assay is not able to differentiate between epimers (i.e. catechin and epicatechin, the building blocks of CT). Stereochemical differences are of major importance in CT biosynthesis, since they affect the formation of the subsequent polymers in the flavonoid pathway (Dixon *et al.* 2005). Both the composition (e.g. proportion of *cis* and *trans* isomers) and degree of polymerization of CT can change with increasing maturity (Koupai-Abyazani *et al.* 1993). It is unknown whether or how the composition of isomers at different stages of development might affect forage palatability, but it appears that stereochemistry at the C-2-C-3 position influences the biological reactivity of condensed tannins (Barry and McNabb 1999; Kraus *et al.* 2003).

It was interesting to observe that the most promising accessions (in terms of high forage quality, i.e. high digestibility, low tannin content) CIAT 18438, CIAT 21083, CIAT 21090 and CIAT 22082 all belong to the semi-erect morphotype M3. They were strikingly different from all other *F. macrophylla* accessions and also from other semi-erect accessions since they had very low extractable CT content, low tannin extractability, tannins with low protein-binding capacity, and high DM digestibility values. For three of these accessions (CIAT 21083, CIAT 21090 and CIAT 22082) the monomer composition of the sCT confirmed that they were also different in terms of procyanidin:prodelphinidin: propelargonidin ratio.

This provides further support to findings of an agronomic evaluation (Chapter 5) that identified accessions belonging to this morphotype as particularly promising in terms of DM production and digestibility. However, high DM digestibility was also detected in various erect accessions (morphotype M1), and in a preliminary "cafeteria" grazing trial semi-erect accessions of morphotype M2 were the most palatable whereas the high digestibility/low tannin accessions selected above were only moderately palatable. The low palatability and low seed production of the selected accessions may limit their dissemination as forage plants.

However, it should be kept in mind that 'palatability' is influenced not only by the chemical composition of a forage - which is influenced by complex genotype x environment interactions - but also by factors related to the consuming animal, e.g. prior learning, time of adjustment to new feeds, different species and even breeds of animals (Faint *et al.* 1998; Shelton 2000). Therefore, palatability of the selected accessions needs to be assessed more

thoroughly in cafeteria trials at different stages of maturity, and also with cut material offered fresh, wilted, and as silage or hay, including adequate adaptation periods for livestock. Also, offering a mixture of a palatable forage species with high-quality accessions of *F. macrophylla* might be an option to overcome these limitations. Furthermore, it is suggested that studies should be carried out to elucidate how differences in quality of the two semi-erect morphotypes M2 and M3 as well as of erect accessions (M1) impact animal production. Attention also needs to be paid to the enhancement of seed production of these accessions, e.g. through evaluations in different environments (e.g. latitudes, altitudes) and micronutrient input.

Conclusions

The results from this study revealed high variability in a representative subset of 23 *F. macrophylla* and one *F. stricta* accessions in terms of IVDMD, fibre and tannin content, tannin monomer composition and tannin protein-binding capacity. Four semi-erect accessions (CIAT 18438, CIAT 21083, CIAT 21090 and CIAT 22082) were the most promising ones because they had high digestibility, tannins with low protein-binding capacity and were virtually free of extractable condensed tannins. They were thus superior to the control accession CIAT 17403 in terms of forage quality and could be an option for livestock production systems in acid infertile soils, for which only few forage shrub legumes of good quality are available.

The identification of some *F. macrophylla* accessions with low tannin content and high digestibility provides the opportunity to select productive low tannin accessions of high nutritive value for future introduction into tropical livestock-based farming systems. However, further evaluations during more than one season and at different developmental stages are needed to verify these results. Furthermore, research is warranted on the nutritive characteristics of a larger set of *F. macrophylla* accessions and different *Flemingia* species (*F. stricta* seems promising), including cafeteria grazing trials. Particular attention should be given to the *F. macrophylla* morphotypes M1 (erect), M2 (semi-erect) and M3 (semi-erect), in order to clarify the effect of extractable CT on digestibility, acceptability and animal production.

Chapter 7

Molecular characterization of a collection of the tropical multipurpose shrub legume *Flemingia macrophylla*

Abstract

Random amplified polymorphic DNA (RAPD) markers were used for assessing genetic diversity and its structure in a collection of the drought-tolerant, tropical multipurpose shrub legume *Flemingia macrophylla* (Willd.) Merrill. The species is especially suited to low-input smallholder production systems and is used as dry season forage supplement, live soil cover, mulch and living barrier, among others. Genetic groups identified by multiple correspondence analysis of RAPD markers related closely to four morphotypes revealed by multivariate analysis of morphological, agronomic and forage quality characteristics. Overall genetic diversity in the collection was moderate (H_T = 0.241), with 79% differentiation among and high genetic similarity (*GS*) within groups (0.672 to 0.965). Results indicate a closer relationship of morphotypes M3 and M4 with *F. stricta* rather than with morphotypes M1 and M2. The morphotype M1 was genetically the most depauperate, followed by morphotypes M2 and M4. Various duplicate accessions ($P < 0.05$) were identified, and evidence is provided that non-Asian accessions are not native to their collection sites, but rather introduced from Southeast Asia.

Keywords: *Flemingia macrophylla*, *Flemingia paniculata*, *Flemingia stricta*, forage legume, genetic diversity, morphotypes, molecular marker, RAPD

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Introduction

The genus *Flemingia* Roxb. ex W.T. Aiton (Leguminosae: Papilionoideae) is one of 13 genera belonging to the subtribe *Cajaninae*, and its species are considered as wild relatives of the important tropical grain legume pigeonpea (*Cajanus cajan* (L.) Millsp.). Phylogenetic relationships between pigeonpea and its wild relatives, including *Flemingia*, have been studied using restriction fragment length polymorphisms (RFLP) and random amplified polymorphic DNA (RAPD) markers (Nadimpalli *et al.* 1992; Ratnaparkhe *et al.* 1995; Lakshmi *et al.* 2000), but the taxonomic status within *Flemingia* is still unclear (Maesen 2003; ILDIS 2005). The genus is currently under revision (L.J.G. van der Maesen, personal communication 2005).

Flemingia macrophylla (Willd.) Merrill (syn. *F. congesta, Moghania macrophylla*) is a drought tolerant multipurpose tropical shrub legume adapted to acid, low-fertility soils. The shrub can grow up to 3 m high and is used as dry season forage supplementation, live soil cover or mulch, erosion barrier hedges, shade-providing shrub in young coffee and cocoa plantations, and as firewood (Andersson *et al.* 2002). The species is reported native to Southeast Asia, and introduced to tropical Africa, Australia and South America (Verdcourt 1979; Hacker 1990).

The main germplasm collection of *F. macrophylla* comprises 85 accessions and is held by the International Centre for Tropical Agriculture (CIAT), Cali, Colombia (IPGRI 2005). Most of the accessions originate from Southeast Asia and few were collected in South America and Africa. Four different morphotypes have been identified in the collection that can be distinguished by flower and seed characteristics, and stem pubescence (Chapter 4, Andersson *et al.* 2005c). Diversity within the collection has also been assessed in terms of agronomic traits (Chapter 5, Andersson *et al.* 2005c) and forage quality (Chapter 6, Andersson *et al.* 2005a), and some promising accessions have been identified for further evaluation. A RAPD study conducted on a set of 37 *F. macrophylla* accessions collected in North Vietnam showed significant differences in genetic diversity between populations from highland and lowland regions (Heider *et al.* 2006).

Knowledge about the extent and distribution of genetic diversity within and among populations is of crucial importance for the conservation and utilization of species in general. Traditionally, the assessment of genetic variability is largely based on agro-ecological characteristics and morphological descriptors which interact with the genetic background and the environment (e.g. Conover and Schultz, 1995; Hoffman and Merilä, 1999). Emphasis is now increasingly placed on the use of molecular methods, such as RFLP, single sequence repeats (SSR), amplified fragment length polymorphisms (AFLP) and RAPDs that allow a more rapid assessment of quantitative genetic diversity and relationships (Rao and Riley

1994; Lee 1998). Among the different molecular marker approaches, each method has its assets and limitations. The choice of the most appropriate marker technique depends not only on the particular objective(s) of the study, but also on the technical requirements and the cost and time investments implied. RAPDs are an effective and relatively inexpensive technique not requiring any prior sequence information and can therefore be applied to a wide range of pant and animal taxa (Tingey and Tufo 1993; Karp *et al.* 1997). They are dominant, neutral markers using random sequences as primers that sample the genome randomly and generate multiple numbers of amplifiable polymorphic fragments, allowing the analysis of a large number of samples in a short time (Williams *et al.* 1990).

It must be acknowledged, however, that single bands on the gel can sometimes be comprised of more than one co-migrating fragment. Also, the presence of putatively identical bands in different individuals cannot be taken as evidence that they are homologous, although they may have the same molecular weight (Tingey and Tufo 1993). In *Brassica*, however, lack of homology has been detected only between species and not within, suggesting that this limitation might be more critical in inter- than in intraspecific relationships (Thormann *et al.* 1994). Another limitation of RAPDs is their low reproducibility (e.g. Jones *et al.* 1997a), but careful adherence to protocols can minimize this drawback (Penner *et al.* 1993; Lowe *et al.* 1996). Furthermore, the dominant nature of RAPDs may lead to an overestimate of allele frequencies necessary for population-genetic analysis, and thus to reduced accuracy relative to analyses with codominant markers (Lynch and Milligan 1994).

RAPDs have been successfully used in molecular studies of tropical tree and shrub legumes, e.g. *Gliricidia sepium* (Jacq.) Walp. (Chalmers *et al.* 1992; Dawson *et al.* 1995), *Caesalpinia echinata* Lam. (Cardoso *et al.* 1998) and *Cratylia argentea* (Desv.) O. Kuntze (Chapter 3), allow the quantification of similarity, or difference, and have their strength in distinguishing individuals, cultivars or accessions (Lee 1995; Karp *et al.* 1996). They have also been used for taxonomy and the assessment of phylogenetic relationships among populations, groups of populations, and species (Demeke *et al.* 1992; Whitkus *et al.* 1994; Sharma *et al.* 1995; Kaga *et al.* 1996). Quantitative phenetic analysis of RAPDs frequently produces matrices of genetic similarity that are consistent with classifications based on morphological and agronomic criteria (Halward *et al.* 1991; Demeke *et al.* 1992; Kazan *et al.* 1993; Tao *et al.* 1993), and empirical data from comparative studies suggest that the degree of genetic differentiation in neutral marker (such as RAPD) loci is closely predictive of the degree of differentiation in loci coding quantitative (e.g., morphological) traits (Merilä and Crnokrak 2001). RAPDs are thus a useful complementary tool to traditional phenotypic approaches, and both methods are needed to accurately assess the diversity of genetic resources.

The objective of the present research was to study variation in RAPD markers to a) assess the genetic diversity in a collection of 69 *F. macrophylla* accessions held by CIAT,

b) exemplarily assess genetic diversity within accessions, c) identify genotype duplicates and taxonomic mismatches in the collection, d) clarify the status of accessions collected in Africa and tropical America, and e) compare the results obtained with RAPDs to those obtained from studies using conventional taxonomic traits to better understand the relationships among the different morphotypes described for the species (Chapter 4).

Materials and methods

Plant material and DNA extraction

Sixty-nine *Flemingia macrophylla* accessions, originating mainly from Southeast Asia, were used in the study (Table 7.1). Five accessions of *F. stricta* and two of *F. paniculata* were included as additional reference to validate the genetic assessment of *F. macrophylla*, expecting the genetic similarity among *F. macrophylla* accessions to be greater than between accessions of *F. macrophylla* and accessions of *F. stricta* or *F. paniculata*.

Accessions are bulk samples representing populations at their respective collection sites. Seeds were germinated and seedlings raised in the greenhouse before transplanting them to the field at the CIAT-Quilichao Experimental Station (Cauca department, Colombia; 03°06'N, 76°31'W; 990 m.a.s.l.). Young leaves were harvested in the field just before reaching fully developed size.

Total genomic DNA was extracted from 50 mg tissue macerated in liquid nitrogen by means of a small-scale DNA extraction method (DNeasy® Plant Mini Extraction Kit, Qiagen Inc., Valencia, CA, USA) with the following modifications: 600 instead of 400 µl Buffer AP1, 2 instead of 4 µl RNAse, and 150 instead of 130 µl Buffer AP2. DNA was quantified using a DyNA Quant™ 200 fluorometer (Hoefer Scientific Instruments, San Francisco, CA, USA) and diluted to a final concentration of 5 ng DNA μl^{-1}.

For the determination of genetic variability within accessions, leaves were harvested of ten individual plants from each of seven accessions representing the different *F. macrophylla* morphotypes (M1: CIAT 801 and CIAT 21529, M2: CIAT 17403, M3: CIAT 21090, M4: CIAT 20975 and CIAT 21990) and of a *F. stricta* reference accession (CIAT 21092). DNA extracted from four individuals of accession CIAT 20975 and seven individuals of accession CIAT 801 was lost due to refrigeration problems. Results obtained from within-accession assessment of genetic diversity showed high differentiation among and little variability within these seven accessions (Table 7.2). It was therefore decided to use bulked samples of only three individual plants per accession for determining genetic variability among accessions.

RAPD analysis

RAPD analysis was performed following the methodology of Welsh and McClelland (1990) and Williams *et al.* (1990). The volume of the final reaction (25 µl) contained 1X PCR buffer (50 m*M* KCl, 10 m*M* Tris-HCl pH 8.8, 0.1% Triton x-100), 2.5 m*M* $MgCl_2$, 0.2 m*M* of each dNTPs, 0.2 µ*M* primer (Series OPD, OPG, OPI and OPJ from Operon Technologies, Alameda, CA, USA), 1 U Taq DNA polymerase (Promega Corp., Madison, WI, USA) and 25 ng of template DNA. A DNA-free control was included in each round of reactions. Amplifications were performed in a thermocycler (PTC-100^{TM}, MJ Research Inc., Watertown, MA, USA) with an initial denaturing step of 5 min at 94 °C followed by 40 cycles of 30 s at 94 °C, 30 s at 38 °C and 1 min at 72 °C and a final extension step of 5 min at 72 °C. The PCR products were run on a 1.4% agarose gel at 20 V cm^{-1} during 45 min and visualised by ethidium bromide staining (0.5 µg ml^{-1} bromide in gel and buffer) under UV-light. Subsequently, they were photographed with a Kodak digital camera DC 120 (Software Kodak Digital Science^{TM} 1997).

Data analysis

Amplified DNA fragments were scored as present (1) or absent (0) for each primer, and variations in band presence were recorded as polymorphisms (Lynch and Milligan 1994). The resulting matrix was used to estimate genetic similarity among all pairs of genotypes by means of the Nei and Li similarity coefficient (Nei and Li 1979), adapted from Dice (1945). The Nei and Li similarity coefficient is based on the proportion of shared fragments: $GS(i,j) = 2N_{ij} / (2N_{ij} + N_i + N_j)$, where N_{ij} is the number of bands shared by both samples, N_i is the number of bands present in *i* and absent in *j*, and N_j is the number of bands present in *j* and absent in *i*. The unweighted pair group method with arithmetic averages (UPGMA) was used to carry out average linkage cluster analysis based on the similarity matrix (Sneath and Sokal 1973). Groups were then analysed based on a level of 85% of genetic similarity. The calculation of genetic similarity and cluster analysis were performed using the NTSYS package version 2.1 (Rohlf 2000).

Furthermore, multiple correspondence analysis (MCA) was performed on the original matrix to provide an additional representation of genetic similarity and to visualize the dispersion of individuals in relation to their molecular profile, based on the first three principal axes of variation. The number of groups (6) was defined considering a value of $R^2 = 0.99$. Means of genetic similarity (Nei and Li 1979) and genetic diversity (Nei 1973) within and among groups were determined for both methods (Cluster analysis and MCA) to analyse the coherence of the resulting classifications and compare them with the grouping into morphotypes based on morphological descriptors. MCA and means of genetic diversity were calculated using SAS version 8.2 (SAS Institute Inc. 1999).

Table 7.1 Geographic origin of 69 *Flemingia macrophylla*, five *F. stricta* and two *F. paniculata* accessions

Accession (CIAT No.)	Country of origin[a]	State/Province	Latitude	Longitude	Altitude (m asl)	Morpho-type[b]
801	n.a.	n.a.	n.a.	n.a.	n.a.	M1
7184	n.a.	n.a.	n.a.	n.a.	n.a.	M1
17400	THA	Northeastern Thailand	16° 41' N	102° 48' E	160	M2
17403	THA	Southern Thailand	10° 04' N	99° 04' E	40	M2
17404	THA	Southern Thailand	09° 25' N	99° 11' E	50	M2
17405	THA	Southern Thailand	08° 59' N	99° 23' E	70	M2
17407	THA	Southern Thailand	08° 18' N	99° 55' E	50	M2
17409	MYS	Peninsular Malaysia	06° 39' N	100° 17' E	70	M2
17411	MYS	Peninsular Malaysia	03° 20' N	102° 26' E	110	M2
17412	MYS	Peninsular Malaysia	05° 46' N	102° 25' E	50	M2
17413	MYS	Peninsular Malaysia	06° 03' N	102° 11' E	30	M2
18048	CHN	Hainan	19° 23' N	109° 06' E	150	M4
18437	IDN	West Sumatra	00° 29' S	100° 52' E	190	M3*
18438	THA	Eastern Thailand	12° 47' N	101° 44' E	40	M3
18440	THA	Eastern Thailand	12° 17' N	102° 29' E	30	M2
19453	PNG	Papua New Guinea	06° 57' S	146° 34' E	1100	M1
19454	PNG	Papua New Guinea	07° 13' S	146° 35' E	650	M1
19457	PNG	Papua New Guinea	06° 02' S	145° 22' E	1630	M1
19797	IDN	West Sumatra	00° 34' N	100° 01' E	180	M2
19798	IDN	West Sumatra	00° 44' S	100° 50' E	190	M2
19799	IDN	Jambi, Sumatra	02° 08' S	102° 02' E	150	M2
19800	IDN	South Sumatra	03° 47' S	103° 36' E	140	M2
19801	IDN	South Sumatra	03° 57' S	103° 25' E	520	M2
19824	IDN	West Sumatra	00° 39' S	100° 36' E	130	M2
20065	IDN	Rote	n.a.	n.a.	70	-
20616	IDN	Aceh, Sumatra	04° 44' N	96° 45' E	690	M2
20617	IDN	Aceh, Sumatra	05° 01' N	96° 42' E	560	M2
20618	IDN	Aceh, Sumatra	05° 24' N	95° 29' E	250	M2
20621	IDN	Aceh, Sumatra	03° 41' N	97° 37' E	1110	M2
20622	IDN	North Sumatra	02° 34' N	98° 32' E	1350	M1
20624	IDN	North Sumatra	01° 11' N	99° 44' E	30	M2
20625	IDN	West Sumatra	00° 15' N	100° 05' E	270	M1
20626	IDN	North Sumatra	02° 46' N	99° 15' E	250	M1
20631	IDN	North Sumatra	03° 06' N	98° 42' E	550	M1
20744	COL	Tolima	03° 43' N	75° 32' W	930	M1
20972	CHN	Hainan	18° 55' N	110° 26' E	50	M4
20973	CHN	Hainan	18° 47' N	110° 20' E	70	M4
20975	CHN	Hainan	18° 49' N	109° 17' E	230	M4
20976	CHN	Hainan	18° 45' N	109° 30' E	330	M4
20977	CHN	Hainan	18° 55' N	109° 28' E	220	M4
20978	CHN	Hainan	19° 10' N	109° 28' E	250	M4
20979	CHN	Hainan	19° 14' N	109° 23' E	370	M4
20980	CHN	Hainan	19° 23' N	109° 06' E	200	M4
20982	CHN	Hainan	19° 30' N	109° 34' E	140	M4
21079	THA	Central Thailand	14° 46' N	99° 27' E	250	M4
21083	THA	Northern Thailand	18° 40' N	97° 56' E	620	M3
21087	THA	Northern Thailand	18° 51' N	98° 52' E	700	-
21090	THA	Northern Thailand	19° 12' N	99° 31' E	550	M3

Accession (CIAT No.)	Country of origin[a]	State/Province	Latitude	Longitude	Altitude (m asl)	Morpho-type[b]
21241	COL	Meta	03° 32' N	73° 46' W	400	M1
21248	GHA	Kumasi	n.a.	n.a.	n.a.	M1
21249	n.a.	n.a.	n.a.	n.a.	n.a.	M1
21519	HAW	Hawaii (USA)	22° N	158° E	20	M1
21529	IDN	Java	07° 45' S	110° 20' E	750	M1
21580	CMR	Centre	03° 52' N	11° 31' E	760	M1
21982	VNM	North Central Coast	16° 24' N	107° 32' E	70	M4
21990	VNM	South Central Coast	15° 28' N	108° 22' E	80	M4
21991	VNM	South Central Coast	15° 24' N	108° 50' E	40	M4
21992	VNM	South Central Coast	14° 51' N	108° 52' E	40	M4
21993	VNM	South Central Coast	14° 24' N	109° 00' E	150	M4
21995	VNM	Central Highlands	12° 25' N	107° 46' E	800	M4
21996	VNM	Central Highlands	11° 56' N	107° 24' E	450	M4
22082	THA	Northeastern Thailand	18° 14' N	103° 10' E	190	M3
22087	THA	Northeastern Thailand	18° 20' N	103° 41' E	160	-
22090	THA	Northeastern Thailand	17° 53' N	104° 15' E	170	M2
22285	VNM	South Central Coast	12° 33' N	108° 59' E	80	M4
22327	VNM	Central Highlands	11° 05' N	106° 36' E	80	M4
C104890	IND	Madhya Pradesh	22° 42' N	78° 42' E	1080	M1
I-15146	CRI	Puntarenas	08° 38' N	83° 10' W	10	M1
J-001	COL	Meta	03° 13' N	73° 15' W	300	M1
Reference accessions:						
21080	THA	Northern Thailand	17° 39' N	99° 08' E	370	*F. stricta*
21086	THA	Northern Thailand	18° 50' N	98° 52' E	490	*F. stricta*
21092	THA	Northern Thailand	20° 26' N	99° 54' E	500	*F. stricta*
21994	VNM	Central Highlands	14° 00' N	108° 13' E	700	*F. stricta*
22058	THA	Northeastern Thailand	17° 04' N	101° 12' E	370	*F. stricta*
21109	THA	Northern Thailand	18° 28' N	97° 57' E	540	*F. paniculata*
21114	THA	Northern Thailand	18° 55' N	100° 16' E	510	*F. paniculata*

[a] CHN, China; CMR, Cameroon; COL, Colombia; CRI, Costa Rica; GHA, Ghana; IDN, Indonesia; IND, India; MYS, Malaysia; PNG, Papua New Guinea; THA, Thailand; HAW/USA, Hawaii/USA; VNM, Vietnam; n.a., not available

[b] Morphotypes according to Chapter 4: M1, erect accessions (stalked racemes with numerous light pink flowers and black seeds); M2, semi-erect accessions with dark pink flowers in densely congested racemes and glabrous or slightly pubescent stems and petioles; M3, semi-erect accessions with less than 20 dark pink or white flowers in very lax racemes which usually do not exceed the length of the petiole; M4, semi-erect accessions with light pink flowers in densely congested racemes and strongly pubescent stems and petioles. For accessions CIAT 20065, CIAT 21087 and CIAT 22087, the morphotype could not be determined due to missing data

* doubtful status (see Chapter 4)

Results

RAPD profiles

Initially, 47 oligonucleotide decamer primers were screened with three *F. macrophylla* accessions (i.e. genotypes). Of these, 17 primers produced smeared or faint bands, and seven (OPJ 02, OPJ 08, OPG 01, OPG 07, OPD 14, OPD 17, OPD 19) gave no amplification products. Fifteen primers, for which high levels of polymorphisms were obtained, were repeated to test for reproducibility and eight primers were identified that detected distinct, clearly resolved and consistently reproducible amplification products. They were selected for the amplification of RAPD sequences and generated a total of 112 scorable fragments across 69 *Flemingia macrophylla* genotypes and the five *F. stricta* and two *F. paniculata* reference accessions (Table 7.3).

On average, 14 scorable fragments were obtained per primer, ranging from five (primer OPD 04) to 21 (primer OPD 15). The band size ranged from 440 to 2500 bp. A RAPD profile generated by primer OPJ 07 is shown in Figure 7.1. Of the 112 fragments detected across all genotypes analysed, 110 (98%) were polymorphic. Excluding *F. stricta* and *F. paniculata*, 86 fragments were detected, 81 (94%) being polymorphic. Nearly half (46%) of the 112 bands were rare (frequency < 0.10), and only 17% were frequent (> 0.50) in all genotypes.

Cluster and multiple correspondence analysis

The dendrogram based on Nei and Li genetic similarity clearly distinguishes the four *F. macrophylla* morphotypes identified in Chapter 4, and *F. paniculata* and *F. stricta* (Figure 7.2). First, *F. paniculata* is separated (Group 6), followed by a group of 20 *F. macrophylla* accessions (Group 1) which is composed of 19 accessions belonging to morphotype M1 (erect, stalked racemes with numerous light pink flowers and black seeds), and CIAT 20065 with undetermined morphotype (due to insufficient morphological data).

Next, a group (Group 5) with four accessions belonging to morphotype M3 (semi-erect, less than 20 dark pink or white flowers in very lax racemes which usually do not exceed the length of the petiole) is split. Accession CIAT 18437 which in the morphological study (Chapter 4) had clustered together with these accessions, was genetically more similar to the accessions of morphotype M2 (semi-erect, dark pink flowers in densely congested racemes and glabrous or slightly pubescent stems and petioles), with which it clustered in Group 2.

Next, the five *F. stricta* accessions were separated (Group 4), followed by Group 3 which comprises the 20 accessions belonging to morphotype M4 (semi-erect, light pink flowers in densely congested racemes and strongly pubescent stems and petioles), as well as accessions CIAT 21087 and CIAT 22087 (morphotype undetermined) and CIAT 22090 which had been classified as morphotype M2 in the morphological study (Chapter 4).

Table 7.2 Nei estimates of genetic diversity (heterogeneity) among and within six *Flemingia macrophylla* and one *F. stricta* accessions

Accession (CIAT No.)	Morpho-type†	*n*	H_i
801	M1	3	0.000
21529	M1	10	0.000
17403	M2	10	0.092
21090	M3	10	0.000
20975	M4	6	0.000
21990	M4	10	0.073
21092	*F. stricta*	10	0.000
H_S			*0.028*
H_T			*0.179*
G_{ST}			*0.844*

* *n*, number of individuals; H_T, total genetic diversity; H_i, genetic diversity within each accession; H_S, average genetic diversity within accessions; G_{ST}, coefficient of genetic differentiation (proportion of total genetic diversity found among accessions)
† Morphotypes M1 to M4, see Table 7.1.

Table 7.3 Oligonucleotide primers employed in RAPD analysis of 69 *Flemingia macrophylla* accessions, their sequence, number of polymorphic (P) and monomorphic (M) bands obtained and percentage of polymorphic bands (% PB)

Primer code	Sequence (5' to 3')	Number of bands (total)		Number of bands (only *F. macrophylla*)	
		P	M	P	M
OPD 01	ACCGCGAAGG	8	0	6	0
OPD 04	TCTGGTGAGG	5	0	2	0
OPD 15	CATCCGTGCT	21	0	15	1
OPI 07	CAGCGACAAG	18	0	14	0
OPJ 04	CCGAACACGG	14	0	9	0
OPJ 06	TCGTTCCGCA	15	2	14	2
OPJ 07	CCTCTCGACA	14	0	12	0
OPJ 12	GTCCCGTGGT	15	0	9	2
Sum		*110*	*2*	*81*	*5*
Total		*112*		*86*	
PB (%)		*98.2*		*94.2*	

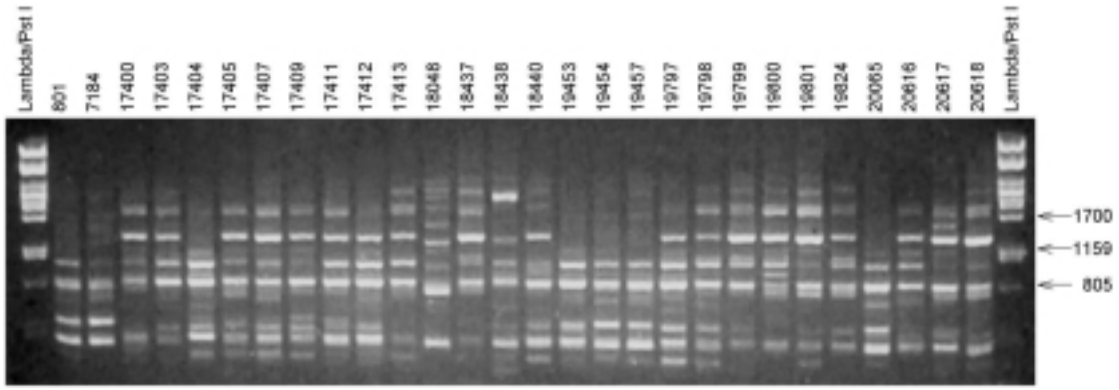

Figure 7.1 RAPD profile obtained using the primer OPJ 07 for *Flemingia macrophylla* genotypes. Size markers (λ-DNA/PstI, Invitrogen, USA) for assessing base pair lengths are shown in the first and last lane

The latter three (accessions CIAT 21087, CIAT 22087 and CIAT 22090) were clearly differentiated from the other 20 Group 3 accessions with Nei-Li coefficients of 0.57 and 0.62 which is close to the value distinguishing *F. stricta*. The accessions belonging to morphotype M3 were differentiated at an even lower Nei-Li coefficient (0.41), suggesting that they are genetically at least as different from the other *F. macrophylla* accessions as is *F. stricta*.

Multiple correspondence analysis confirms the separation and composition of Groups 1 (morphotype M1), 2 (morphotype M2) and 6 (*F. paniculata*) (Figure 7.3). The latter is clearly separated from all other accessions by the second dimension, while dimension 3 visualizes the separation of morphotype M2. Results of the MCA indicate a closer relationship of the *F. macrophylla* morphotypes M3 (Group 5) and M4 (Group 3) with *F. stricta* rather than with the other *F. macrophylla* accessions belonging to the morphotypes M1 and M2. Three accessions (CIAT 21087, CIAT 22087 and CIAT 22090) were not conclusively assigned to any of the four groups of morphotypes mentioned above (Figure 7.3B).

Genetic diversity within accessions

The average genetic diversity within the seven accessions was very low (H_S = 0.028) and represented only 16% of the total genetic variation (Table 7.2). Mean genetic similarity *GS* was 0.506 and ranged from 0.154 to 1.0 within accessions. All individuals of the two accessions CIAT 801 and CIAT 21529, both of which belong to the same morphotype (M1), were genetically identical, as were those of accessions CIAT 20975 (M4) and CIAT 21092 (*F. stricta*). Individuals of the two accessions CIAT 21990 and CIAT 20975 (both morphotype M4) were genetically very similar and clustered together closely in MCA (data not shown).

Genetic diversity among and within species and groups

The mean genetic diversity in the entire collection was H_T = 0.241, with high differentiation among groups (G_{ST} = 0.792) (Table 7.4). Genetic diversity was very heterogeneously distributed, and was highest within Groups 4 and 5 (H = 0.140 and 0.150, respectively). Group 1 (morphotype M1) was genetically the most depauperate (H = 0.017), followed by Groups 2 and 3 (morphotypes M2 and M4; both H = 0.040).

The mean genetic similarity was moderate (*GS* = 0.519), ranging from 0.018 to 0.448 among groups (Table 7.5, above diagonal) and from 0.136 to 1.000 among accessions. If a particular group is genetically well differentiated from other groups, then the mean genetic similarity among accessions within the group should be greater than among groups. This was particularly true for *F. paniculata* (Group 6) and for the *F. macrophylla* morphotype M1 (Group 1), which had a *GS* greater than 0.95. The mean genetic similarity within morphotypes

M2 (Group 2) and M4 (Group 3) was also very high (*GS* > 0.90). Groups 4 and 5 were the most different with *GS* = 0.672 and 0.674, respectively.

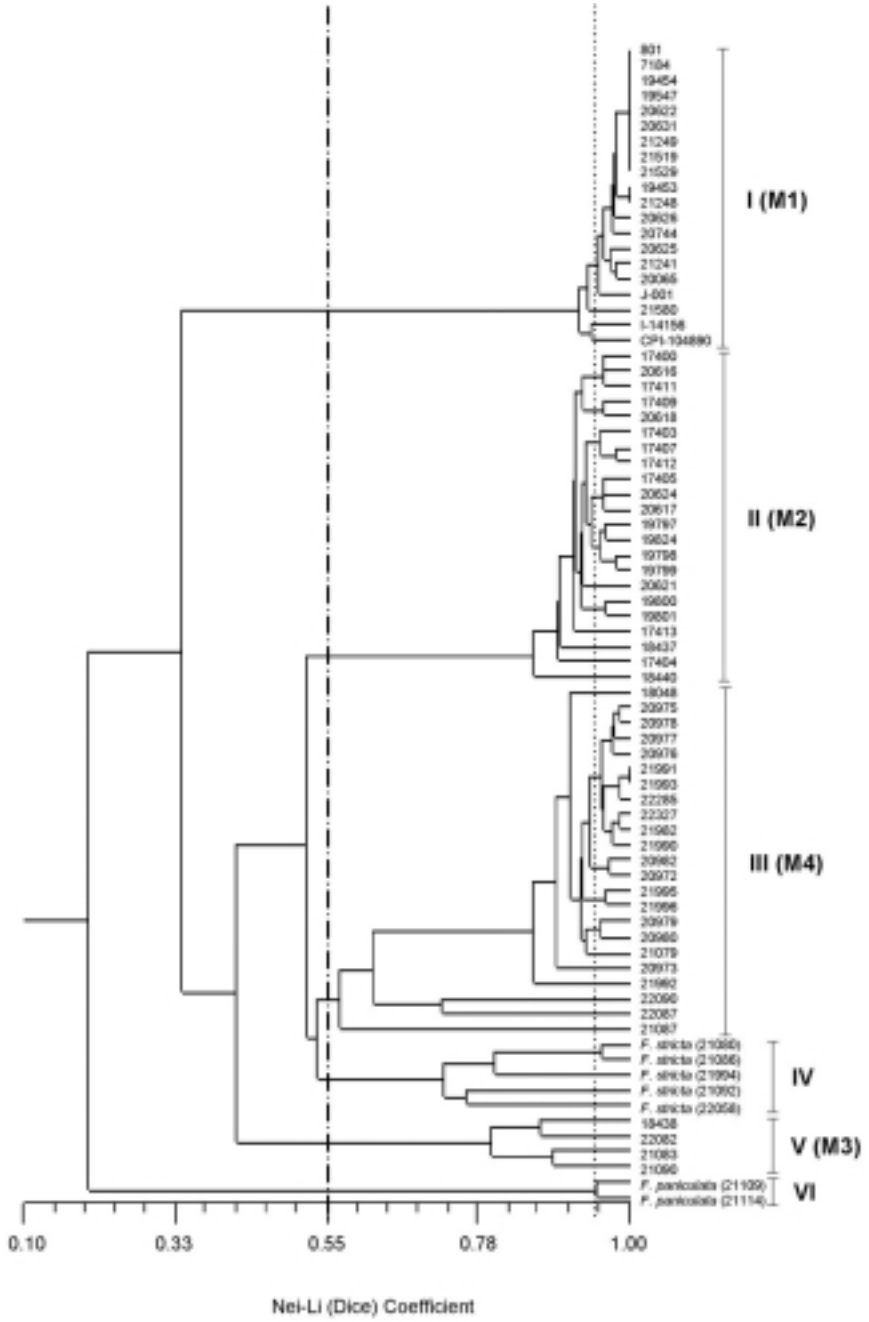

Figure 7.2 Grouping of 69 *Flemingia macrophylla*, five *F. stricta* and two *F. paniculata* accessions using UPGMA cluster analysis. Genetic distances are according to Nei and Li based on RAPD marker. CIAT accession number and morphotype (see Table 7.1) are given below group numbers, as well as the geographic origin of non-Asian accessions. Dash-dotted line: level of six groups; dotted line: level of 95% genetic similarity

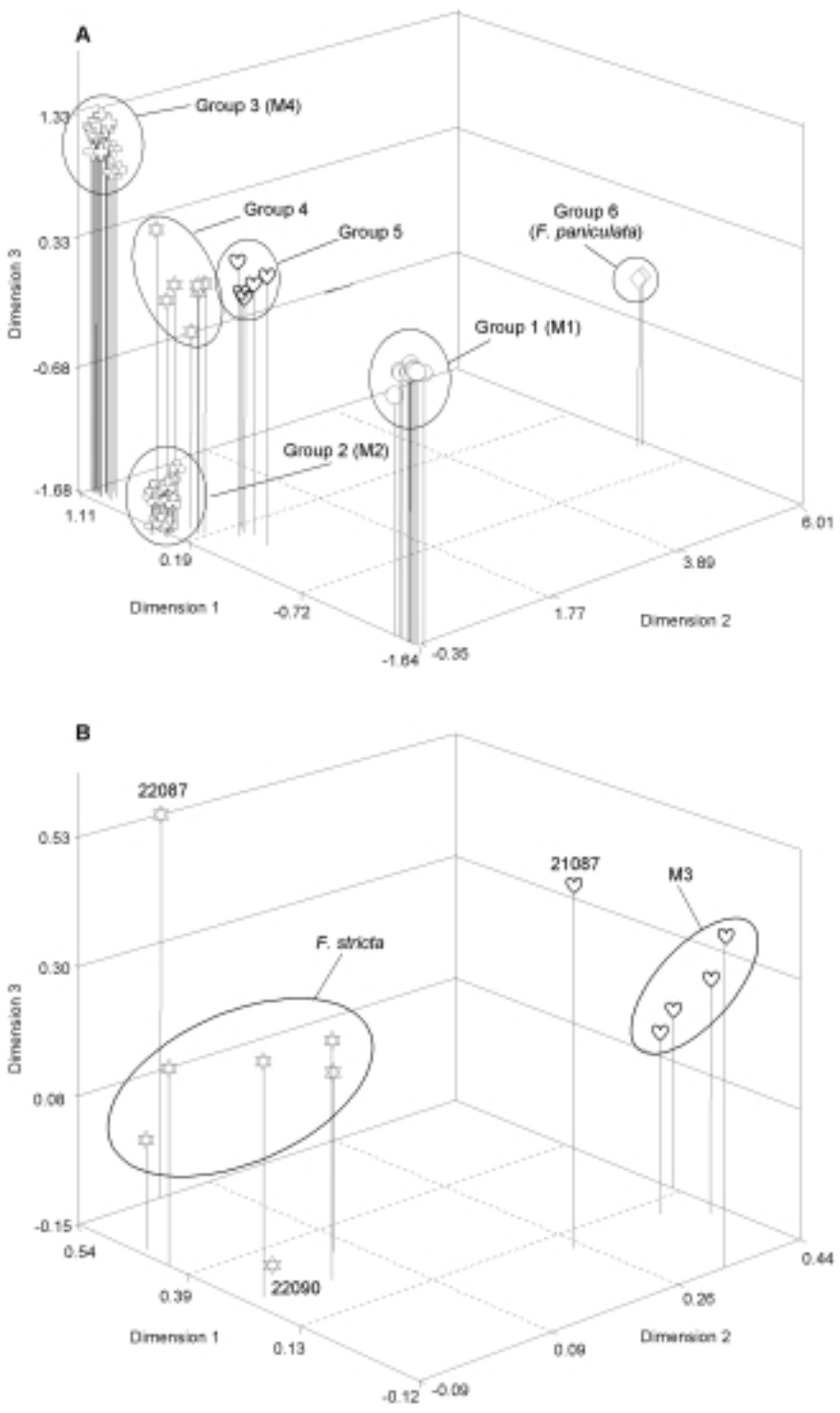

Figure 7.3 Three-dimensional representation of six groups derived from multiple correspondence analysis of RAPD markers among 69 *Flemingia macrophylla*, five *F. stricta* and two *F. paniculata* accessions (A). (B) shows a higher resolution of Groups 5 (hearts) and 6 (stars). M1 to M4, morphotypes 1 to 4 (see text)

Table 7.4 Nei estimates of genetic diversity (heterogeneity) among groups of *Flemingia macrophylla* germplasm

Group[†]	*n*	H_i
1	20	0.017
2	22	0.040
3	20	0.040
4	7	0.140
5	5	0.150
6	2	0.026
H_S		*0.050*
H_T		*0.241*
G_{ST}		*0.792*

n, number of accessions; H_T, total genetic diversity; H_i, genetic diversity within each group; H_S, average genetic diversity within groups; G_{ST}, coefficient of genetic differentiation (proportion of total genetic diversity found among groups)
[†] Groups according to multiple correspondence analysis (Figure 7.3)

Table 7.5 Genetic similarity among (above diagonal) and within (diagonal) groups of *Flemingia macrophylla* germplasm, five *F. stricta* and two *F. paniculata* accessions, based on all pairwise similarities between accessions according to molecular marker information (RAPD)

Group*	N	1	2	3	4	5	6	Total
1	20	0.965	0.296	0.200	0.224	0.290	0.131	
2	20		0.918	0.448	0.390	0.375	0.067	
3	22			0.929	0.435	0.423	0.067	
4	2				0.672	0.314	0.018	
5	5					0.674	0.167	
6	7						0.951	
Total								*0.519*

* Groups according to multiple correspondence analysis (Figure 7.3)

Table 7.6 Descriptive statistics of the loci identified by random amplified polymorphic DNA (RAPD) analysis characterizing 69 *Flemingia macrophylla*, five *F. stricta* and two *F. paniculata* accessions

Group[a]	*n*	Loci				
		Total no.	M	P	% P	Group-specific loci
1	20	34	23	11	32	6
2	22	37	18	19	51	2
3	20	43	21	22	51	2
4	7	52	10	42	81	-
5	5	47	10	37	79	-
6	2	32	29	3	9	16

[a] see Figure 7.2; *n*, number of accessions; groups according to multiple correspondence analysis (Figure 7.3)

Different degrees of polymorphism were detected within groups (Table 7.6). The highest number of scorable markers was obtained for Group 4 (81% being polymorphic), and the lowest for Group 6 (*F. paniculata*), with only 9% of them being polymorphic. The latter, however, is probably due to the low sample size of this group (only two accessions). Species- and group-specific markers were amplified: Sixteen unique markers were detected for *F. paniculata* and two for *F. stricta*. Two markers were present in all *F. macrophylla* morphotypes and in *F. stricta*, but absent in *F. paniculata*. No marker was detected unique only to *F. macrophylla*. When excluding *F. stricta* and *F. paniculata*, six markers generated by primer OPD 04, OPD 15 and OPJ 06 were unique to Group 1 (morphotype M1), two to Group 2 (morphotype M2: OPD 15 and OPI 07), and one marker was unique to Group 3 (morphotype M4: OPJ 06). Six markers (OPD 15, OPI 07, OPJ 07 and OPJ 12) were unique to the four accessions of morphotype M3 belonging to Group 5. One marker was unique to accession CIAT 21087, and two to CIAT 22087.

Identification of duplicates

Nine identical genotypes were detected which could not be discriminated on the basis of the RAPD products scored in this study: accessions CIAT 801, CIAT 7184, CIAT 19454, CIAT 19457, CIAT 20622, CIAT 20631, CIAT 21249, CIAT 21519 and CIAT 21529 shared 100% of their bands ($GS = 1.00$). Accessions CIAT 19453 and CIAT 21248, and CIAT 21991 and CIAT 21993 also paired together at 100% similarity (Figure 7.2). Furthermore, all accessions belonging to morphotype M1 except CIAT 21580, I-15146 and CPI-104890 are not significantly different ($P > 0.05$) from each other, if considering genotypes as duplicates when their pairwise genetic similarity *GS* is equal to or greater than 0.95 (Figure 7.2, Table 7.7).

Similarly, many accessions within morphotypes M2 and M4 are duplicates at $P < 0.05$, as well as the *F. paniculata* accessions CIAT 21109 and CIAT 21114 and the *F. stricta* accessions CIAT 21080 and CIAT 21086.

Status of non-Asian accessions

All seven non-Asian accessions belong to morphotype M1 and clustered together with the Asian M1 accessions in group I. Five of them (CIAT 20744, CIAT 21241 and J-001 from Colombia, CIAT 21519 from Hawaii and CIAT 21248 from Ghana) are genetic duplicates ($P < 0.05$) of a group of 12 Southeast Asian accessions, including the commercial accessions CIAT 801 and CIAT 7184 (Table 7.7). The remaining two non-Asian accessions CIAT 21580 from Cameroon and I-14156 from Costa Rica were genetically similar to this group at a level of over 90%.

Table 7.7 Duplicates within *Flemingia macrophylla* morphotypes, *F. stricta* and *F. paniculata*, based on the Nei and Li coefficient of genetic similarity ($P < 0.05$)

Morphotype/ species*	Accession (CIAT No.)	
M1	• 801, 7148, 19453, 19454, 19457, 20065, 20622, 20625, 20626, 20631, 20744 (COL), 21241 (COL), 21248 (GHA), 21249, 21519 (HAW), 21529, J-001 (COL)	• 21580 (CMR) • I-14156 (CRI) • CPI-104890
M2	• 17400, 17411, 20616 • 17409, 20618 • 17403, 17407, 17412 • 17405, 20617, 20624 • 19797, 19798, 19799, 19824 • 19800, 19801	• 17413 • 17404 • 18437 • 18440 • 20621
M3	• 18438 • 21083	• 21090 • 22082
M4	• 20975, 20976, 20977, 20978, 21982, 21990, 21991, 21993, 22285, 22327 • 20972, 20982 • 20979, 20980	• 21995, 21996 • 18048 • 20973 • 21079 • 21992
F. stricta	• 21080, 21086 • 21092	• 21994 • 22058
F. paniculata	• 21109, 21114	

* Morphotypes M1 to M4, see Table 7.1. CMR, Cameroon; COL, Colombia; CRI, Costa Rica; GHA, Ghana; HAW, Hawaii/USA

Discussion

Polymorphisms

The level of polymorphism detected in *Flemingia* with RAPD markers was very high (> 90%), with an average of 14 polymorphic bands per primer, and similar to that reported for a collection of 37 *F. macrophylla* accessions from Vietnam (95%, Heider *et al.* 2006). RAPD studies with pigeonpea (*Cajanus cajan* (L.) Millsp.) (Ratnaparkhe *et al.* 1995; Souframanien *et al.* 2003) and *Cratylia argentea* (Desv.) O. Kuntze (Chapter 3) (*Papilionoideae*, subtribes *Cajaninae* and *Diocleinae*, respectively) yielded similarly high levels of polymorphism.

Genetic structure within the collection and taxonomic implications

Species delimitation within *Flemingia* which comprises about 40 species, is not clear (Maesen 2003; ILDIS 2005) and the genus is currently under revision. RAPD data allow the quantification of similarity, or difference, and have their strength in distinguishing individuals, cultivars or accessions (Lee 1995; Karp *et al.* 1996). They have also been used

for taxonomy and the assessment of phylogenetic relationships among populations, groups of populations, and species (Demeke *et al.* 1992; Whitkus *et al.* 1994; Sharma *et al.* 1995; Kaga *et al.* 1996).

It must be acknowledged, however, that single bands on the gel can sometimes be comprised of more than one co-migrating fragment. Also, the presence of putatively identical bands in different individuals cannot be taken as evidence that they are homologous, although they may have the same molecular weight (Tingey and Tufo 1993). In *Brassica*, however, lack of homology has been detected only between species and not within, suggesting that this limitation might be more critical in inter- than in intraspecific relationships (Thormann *et al.* 1994). In general, quantitative phenetic analysis of RAPD often produces matrices of genetic similarity that are consistent with classifications based on morphological and agronomic criteria (Halward *et al.* 1991; Demeke *et al.* 1992; Kazan *et al.* 1993; Tao *et al.* 1993).

Both cluster analysis based on Nei and Li's genetic similarity as well as MCA provide evidence for the existence of distinct groups within *F. macrophylla*. Most accessions were precisely assigned to the morphotype determined by conventional morphological and phenological evaluation, and the genetic groups correspond closely to those revealed by multivariate analysis of morphological (Chapter 4) and agronomic (Chapter 5) characteristics following measurements in the field (Andersson *et al.* 2005c).

Overall genetic diversity in the collection was moderate, 79% of it being due to differentiation among groups. Genetic diversity within groups was considerably lower, and the morphotypes M1, M2 and M4 were genetically depauperate. The low genetic diversity within morphotypes indicates isolation of these groups which can be caused by geographical, biological or genetic barriers (Frankel *et al.* 1995). Given the little knowledge about *F. macrophylla* and the confusion about its taxonomic status, discussion of the reasons for the low genetic diversity observed within morphotypes can only be speculative.

For example, clustering did not reflect the geographical origin of accessions, although some geographical tendencies could be observed: all accessions belonging to morphotype M4 were collected in China (Hainan) and Vietnam, and one accession (CIAT 21079) originate from Central Thailand, while M3 and *F. stricta* accessions originate exclusively from Thailand. These tendencies, however, are rather subtle, and in general the distribution ranges of the four morphotypes overlap widely. For example, accessions of the most differentiated group M1 with the lowest within-group diversity were collected throughout Southeast Asia, from Northern India to Papua New Guinea. It thus seems that isolation is not due to geographical barriers.

The overlap of flowering times of the four morphotypes at the experiment site in the Cauca valley, Colombia, has been discussed earlier (Chapter 4). It has to be acknowledged, however, that flowering times at the sites of origin may be different, so that the possibility of population isolation due to biological (temporal) barriers cannot be excluded. Studies with forced crossing between individuals of the same as well as of different morphotypes should be carried out to determine if intraspecific genetic outcrossing barriers exist between morphotypes.

Mating system

The low genetic diversity and low level of polymorphism detected within groups/morphotypes (*Hs* = 0.241) as well as within accessions (*Hs* = 0.179) is in concordance with a predominantly self-pollinating nature of the species. On the other hand, as mentioned above, outcrossing among morphotypes cannot be excluded. Generally, tropical legumes particularly of the *Phaseoleae* tribe are predominantly self-pollinating (Kalin Arroyo 1981; Hacker and Hanson 1999; Schultze-Kraft and Keller-Grein 1999), although cross-pollination seems often to be more common than expected (Cameron and Irwin 1986; Saxena *et al.* 1990). In order to conclusively determine the mating system of *F. macrophylla*, outcrossing rates should be established either by genetic analysis with codominant markers or, less cost-intensive, morphologically. White flower colour has been found in accession CIAT 22082 (M3) and could be used as a recessive marker to establish the proportion of coloured-flowered off-types (Miles 1985; Maass and Torres 1998) in case no genetic isolation barriers between morphotypes exist.

Duplicates in the collection

Strictly, two samples could only be termed genetically identical after comparison of their entire genomes. On the other hand, however, samples can be considered redundant if a major part of their genome coincides. Pejic *et al.* (1998) found that 150 RAPD markers were sufficient for reliable estimates of genetic similarity in maize. Virk *et al.* (1995) reported that differences between very similar pairs of rice accessions can be detected with 99% confidence when examining a total of 86 RAPD markers, and suggested as a rational general strategy to designate accessions as duplicates if they showed no variation across 100 RAPD markers. Based on this assumption, the 13 genotypes detected within the M1 and the M4 morphotypes which could not be discriminated on the basis of the 112 RAPD markers scored in this study, are considered as genetically identical (100% similarity) and thus duplicates.

Furthermore, various groups of accessions within morphotypes M1, M2 and M4 as well as within *F. stricta* and *F. paniculata* were identified as duplicates at $P < 0.05$ (Table 7.7). In terms of agronomic traits and forage quality, however, variability within morphotypes was high (Chapter 5), even within the large group of genetic duplicates identified within

morphotype M1. For example, dry matter (DM) production among these accessions ranged from 93 to 508 g plant^{-1}, and *in vitro* dry matter digestibility (IVDMD) from 379 to 476 g kg^{-1} (Chapter 5).

It is suggested not to bulk the accessions identified as genetic duplicates but rather to maintain them as individual genotypes in order not to risk the loss of any of the genes or gene complexes conferring important agronomic traits. In addition, a core collection should be created for further evaluations, selecting entries based on the information presented here so that they optimally represent the genetic diversity of the entire collection (Frankel 1984; Brown 1989a). Using a core collection for further characterization and evaluation purposes (e.g. screenings, multilocational trials) as well as for distribution would considerably diminish costs and allow a more efficient management of the holdings.

Status of non-Asian accessions

F. macrophylla is considered not to be native to Africa and tropical America (Verdcourt 1979; Hacker 1990). Five out of the seven non-Asian accessions in the collection were genetically identical to a group of Southeast Asian accessions, including commercial accessions obtained from development projects, and the remaining two were genetically more than 90% similar. These findings provide new genetic evidence that the non-Asian accessions are indeed not native but rather naturalized, proceeding from introduced Southeast Asian accessions.

Status of doubtful accessions

Accession CIAT 18437 had been assigned to morphotype M3 in the morphological study. Field and herbarium observations, however, indicated that it might rather belong to the M2 morphotype (Chapter 4). Molecular marker analysis confirmed this hypothesis by clearly grouping CIAT 18437 together with the other M2 accessions.

Accession CIAT 20065 which had not been included in the morphological field trial due to lack of adaptation (Chapter 4), was assigned to morphotype M1 by genetic analysis. Analysis of herbarium material, however, suggests that the accession should be classified as morphotype M2 (sessile, congested inflorescences, glabrous to slightly pubescent stems and petioles).

No clear assignment to a taxonomic group was obtained for a total of three accessions, based on genetic analysis: CIAT 21087 and CIAT 22087 (for which no morphological information was available), and CIAT 22090 (classified as M2 based on morphological information) were grouped together with M4 accessions in the phenogram derived from clustering based on Nei and Li genetic similarity (Figure 7.2). Multiple correspondence analysis, however, placed these three accessions more closely to *F. stricta* and the M3 morphotype (Figure 7.3B).

This might be due to the fact that MCA is based on the principle of distributional equivalence which assures invariance in the results when identical individuals (here: duplicates within M4) are merged (Johnson and Wichern 1992; Lebart *et al.* 1995). Furthermore, MCA highlights differences by giving more weight to rare bands, such as the unique markers detected in accessions CIAT 21087 and CIAT 22087.

According to its plant height:plant diameter ratio (1.0) and other agronomic and forage quality data, accession CIAT 21087 had been grouped together with the accessions of morphotype M3 (Chapter 5). This was supported by MCA of RAPD data, but not by the Nei and Li coefficient of genetic similarity. Analysis of herbarium material, on the other hand, indicates that this accession might actually be *F. stricta*. Accession CIAT 22087 was grouped together with morphotype M4 by cluster analysis of agronomic and forage quality data (Chapter 5) which was confirmed by clustering based on the Nei and Li coefficient of genetic similarity. Analysis of herbarium material, too, indicated that this accession belongs to morphotype M4. Multiple correspondence analysis of molecular marker data, however, suggests that this accession is genetically at least as similar to *F. stricta* as it is to morphotype M4 (Figure 7.3A). CIAT 22090 had been classified as morphotype M2 based on morphological information (Chapter 4). Genetic analysis gave contradictory results, grouping it together with morphotype M4 on the one hand (Nei and Li coefficient of genetic similarity, Figure 7.2), and with *F. stricta* on the other hand (MCA, Figure 7.3B). No useful herbarium material was available for verification of inflorescence characteristics.

In order to conclusively determine the taxonomic status of these accessions, seeds of accessions CIAT 20065, CIAT 21087, CIAT 22087 and CIAT 22090 are being germinated in the greenhouse at CIAT to verify the morphological traits of these accessions and prepare valid herbarium samples for identification through an expert.

Conclusions

The results demonstrate that RAPD technology provides an effective tool for germplasm analysis in *Flemingia*. The evidence from multiple correspondence analysis of RAPD variation presented here supports the recognition of different taxa within *F. macrophylla*, in line with results from morphological (Chapter 4), agronomic and forage quality evaluations (Chapter 5). However, given the current taxonomic uncertainties in the genus, more detailed morphological analysis of the aggregates is required, in particular of the genotypes classified as morphotype M3 and of the doubtful accessions CIAT 21087, CIAT 22087 and CIAT 22090.

The morphotypes M1, M2 and M4 were shown to be genetically depauperate, with very low genetic diversity within groups as well as within accessions. Particularly, 17 of the 20 morphotype M1 accessions were genetically identical. Using the information presented

here for creating a core collection would considerably reduce the costs of further evaluations and germplasm maintenance. These resources could be more effectively used for future collection missions in order to enhance the *ex situ* collection genetically. Areas with potentially high genetic diversity could be identified by the use of geographic information systems.

Non-Asian accessions from Africa and tropical America were shown to be genetically identical or very similar to commercial material obtained in Southeast Asia, indicating that they derive from introduced material from Southeast Asia.

Chapter 8

Core collection approaches for perennial wild tropical legumes using *Flemingia macrophylla* as an example

Abstract

Genebank management including multiplication of perennial multipurpose tree and shrub legumes is expensive and time-consuming. It is particularly complicated for wild tropical legume species, due to the lack of information, e.g. about flowering induction and reproduction system. Core collections can facilitate a more time- and resource-efficient germplasm management. They can be used for initial characterizations and evaluations to learn about the species' variability, and are a good starting-point for the identification of accessions with particular traits in the reserve collection. Concepts are required for the most appropriate and resource-efficient way(s) of identifying core collections. Using *Flemingia macrophylla* (Willd.) Merrill as example, various core collections were established based on different approaches to assess diversity (morphological, agronomic, genetic and by geographic origin). The individual approaches were compared with each other and with a randomly sampled control core collection, and their representativeness was validated. Core collections based on molecular markers best captured diversity in the entire collection, followed by those based on morphological data and random sampling. The core collections should be further validated in multilocational trials and with more traits. Similar studies are needed to test the applicability of these results to other wild legume species.

Keywords: core collection, methodology, representativeness, validation

Introduction

Core collections have become an effective and accepted tool to improve access and utilization of germplasm collections, meeting the challenge of growing sizes and numbers of collections (Brown *et al.* 1989). The Global Action Plan for the Conservation and Sustainable Utilization of Plant Genetic Resources for Food and Agriculture (FAO 1996) recommends the development of core collections as one of the activities required to enhance the use of plant genetic resources.

According to the original definition, a core collection is a limited set of accessions derived from a germplasm collection, "representing with minimum repetitiveness the genetic diversity of a crop species and its relatives" (Frankel 1984). Frankel and Brown (1984) and Brown (1989a; b) further developed the concept which has since evolved into a widely applied method. The term "core collection" is now defined in a wider sense as a "set of accessions which optimally represents specified genetic diversity" (Hintum 1999).

Thus, according to practical requirements, a core collection can represent the diversity in a complete genus including wild species – e.g., the entire genepool of *Hordeum* (Knüpffer and Hintum 1995) – or only a small part of the known genepool, such as the cultivated *Brassica oleracea* in European collections (Boukema *et al.* 1997) or, for example, a set of lentil accessions from Chile, Greece and Turkey (Erskine and Muehlbauer 1991).

The procedure for creating a core collection typically consists of five steps (Hintum *et al.* 2000): 1) Definition of the 'domain', i.e. the collection or part of the collection to be represented; assembly of passport and other relevant data; 2) decision on the size of the core (usually between 5 and 20% of the domain); 3) grouping: dividing the domain into categories which are expected to be as genetically distinct as possible, e.g., because of differences in particular traits (taxonomy, distribution, phenology, morphophysiology, molecular marker); variation between groups should be maximized, and variation within groups minimized; 4) allocation of accessions: deciding on the number of entries per group; and 5) choice of entries: deciding which entries per group are to be included in the core.

A global survey of genebanks, conducted by the International Plant Genetic Resources Institute (IPGRI), gives an account of more than 60 core collections in 15 countries, involving 51 different crop and pasture species (Brown and Spillane 1999). Grouping strategies used to create these cores were mainly geographic origin of accessions (95%) and morphological traits (77%), while genetic markers were applied only in 10%.

The representativeness of core collections has been verified by various authors (Diwan *et al.* 1994; Balfourier *et al.* 1999; Jackson *et al.* 1999; Tohme *et al.* 1999; Xiurong *et al.* 2000;

Grenier *et al.* 2001). These validations were usually based on the comparison of means and variances of traits. However, using only the mean (or variance) of quantitative traits to evaluate core quality (i.e. representativeness) is a potential pitfall since this measurement does not indicate whether all extremes are represented (Skinner *et al.* 1999). The range is a better criterion to analyse core quality (Holbrook *et al.* 1993; Galwey 1995).

The genebank management of perennial multipurpose tree and shrub legume collections requires expensive and time-consuming seed regeneration processes due to long establishment periods (from a few weeks to several months) and their perennial nature (seed production in the first year often very low or lacking). Furthermore, most of these species are not cultivated but wild (Clements 1996; Schultze-Kraft and Peters 1997). The germplasm collections of wild tropical legumes usually do not comprise more than 50 accessions per species (SINGER 2005), and the lack of information about genetic diversity, population structure, reproduction system, flowering induction, pollination agents and seed production of wild species further complicates their management and multiplication. For example, a lack of knowledge about genetic diversity in the collection restrains the identification of duplicates, and the lack of information about factors related to seed production (e.g. reproduction system, flowering induction, pollination agents) may render multiplication efforts fruitless.

Core collections can facilitate germplasm management of these species and help to use time and resources more efficiently. They allow curators to set priorities for germplasm management, e.g. germination tests and regeneration (Brown 1995; Jackson *et al.* 1999). For breeders, the screening of core collections gives an estimate of the variability in the entire collection. The information obtained is a good starting-point for the subsequent identification of accessions with similar characteristics in the reserve collection, e.g. for the detection of particular traits and for further evaluation (Miklas *et al.* 1999). Concepts are therefore required regarding the most appropriate and resource-efficient way(s) of identifying core collections of wild tropical legumes.
So far, published experiences seem to be limited to one example, *Desmodium ovalifolium* Wall. where a core collection was created on the basis of collection site information in connection with some preliminary evaluation data from one experimental site (Schultze-Kraft and Benavides 1988; Schmidt *et al.* 1997). Different approaches to assess diversity (morphological, agronomic, genetic or by geographic origin) do not necessarily capture the same variability due to complex genotype x environment interactions.

Particularly in the case of *Flemingia macrophylla* (Willd.) Merrill, genetic diversity assessed by random amplified polymorphic DNA (RAPD) reflected morphological and agronomic variability only to a limited extent (Chapter 7). This is probably due to the fact that molecular markers capture variability predominantly at neutral sites in the genome which are usually not correlated or linked to variance at the phenotypic level (Jones *et al.* 1997b). These limitations

must be kept in mind when using molecular markers to verify if a core collection adequately represents the genetic diversity of the entire collection.

The objective of the work presented here is,

a) to test and compare different approaches for identifying core collections of the perennial, wild shrub legume *F. macrophylla* based on similarity regarding
   i) geographic origin,
   ii) morphological and phenological traits,
   iii) agronomic traits and forage quality, and
   iv) molecular markers,

   taking into consideration the practical implications (time and cost efficiency) of each of the aforementioned approaches; and
b) to validate the representativeness of these core collections by comparing the diversity captured with that of the entire collection.

Materials and methods

Experimental data

The data used for the present study pertain to a collection of 70 *Flemingia macrophylla* accessions held at the Genetic Resources Unit of the International Centre for Tropical Agriculture (CIAT), which has been evaluated in field trials between 2000 and 2002. The accessions had been collected mainly in Southeast Asia; few accessions, apparently derived from introduced Southeast Asian commercial material (Chapter 4), come from South America and Africa.

The characteristics of the evaluation site and the experimental design are described in detail in Chapter 4. In the evaluations, four different morphotypes were identified that can be distinguished by flower and seed characteristics, and stem pubescence (Chapter 4, Andersson *et al.* 2005b). Diversity within the collection was also assessed in terms of agronomic and forage quality traits (Chapter 5, Andersson *et al.* 2005c). A molecular marker analysis with random amplified polymorphic DNA (RAPD) confirmed the diversity pattern revealed by the aforementioned studies and showed that overall genetic diversity in the collection was high (Chapter 7). To test and compare different approaches for identifying core collections, the following four data sets were used:

i) Data set 1: Geographic origin

Latitude, longitude, altitude and climatic data (see below) were used to cluster accessions of Southeast Asian origin into biogeographically similar groups, using the computer program FloraMap™ (Jones and Gladkov 2001) and assuming that the climate at collection site is representative of the climatic range of the species (Jones *et al.* 2002). Climatic data used by

FloraMap™ are derived from observations at meteorological stations and correspond to 10-min grid cells (approximately 18 x 18 km at the equator). The climatic variables included are the monthly averages of temperature, rainfall, and diurnal temperature range.

Mean temperature is standardized with elevation using the NOAA TGP-006 digital elevation model (NOAA, 1988) and a lapse rate model (Jones 1991). To provide representative values for each grid square, data are interpolated by means of a simple interpolation algorithm based on the inverse square of the distance between stations and the interpolated point, using the five nearest stations in the inverse distance equation (Jones *et al.* 2002).

Clustering was applied only to the 59 Southeast Asian accessions, since FloraMap™ can only handle climate data from one continent at the time. A set of seven non-Asian accessions and three accessions of unknown origin was treated as a separate group. Entries for the core collection were selected proportionately from this group and from each of the three main Southeast Asian clusters.

ii) Data set 2: Morphological and phenological traits

The data of five morphological and phenological traits selected in Chapter 4 (time to 50% flowering, plant height, leaf area, seed yield and seed weight, Table 8.1) were subjected to clustering (Ward's method).

Table 8.1 Morphological, phenological, agronomic and forage quality attributes used for the different approaches of creating a core collection in *Flemingia macrophylla*

	Unit of assessment	Time of assessment
Morphological and phenological traits[a]		
1. Time to 50% flowering	no. of days after sowing	at 50% of buds flowering
2. Plant height	cm	14 months after sowing
3. Leaf area	cm^2	14 months after sowing
4. Seed yield	g $plant^{-1}$	after cutting
5. Seed weight	g 1000 $seeds^{-1}$	after cutting
Agronomic and forage quality traits[b]		
1. Dry matter (DM) production	g $plant^{-1}$	after 8 weeks of regrowth, in wet season
2. *in vitro* DM digestibility (IVDMD)[c]	g kg^{-1}	
3. Crude protein (CP)[d] content	g kg^{-1}	
4. Regrowth capacity	no. of shoots $plant^{-1}$	
5. Dry matter (DM) production	g $plant^{-1}$	after 8 weeks of regrowth, in dry season
6. *in vitro* DM digestibility (IVDMD)[c]	g kg^{-1}	
7. Crude protein (CP)[d] content	g kg^{-1}	

[a] average of three plants; see **Chapter 4**
[b] average of three plants and three replications; see **Chapter 5**
[c] Two-stage technique (Tilley and Terry 1963; modified by Harris 1970)
[d] Kjeldahl procedure using a conversion factor of 6.25 (AOAC 2003)

iii) Data set 3: Agronomic and forage quality characteristics

The data of seven agronomic and forage quality traits selected in Chapter 5 (dry matter (DM) production, *in vitro* DM digestibility (IVDMD) and crude protein (CP) content in the wet and dry season, and regrowth capacity in the wet season, Table 8.1) were subjected to clustering (Ward's method).

iv) Data set 4: Molecular marker (RAPD) similarity

A similarity matrix based on presence/absence of banding patterns was obtained by RAPD analysis as described in Chapter 3. Assuming that each band position represents a genetic locus with two allelic states (band presence or absence), pairwise genetic similarities among genotypes were calculated using the Nei and Li similarity coefficient, based on the proportion of shared alleles (Nei and Li 1979, adapted from Dice 1945): $GS(i,j) = 2N_{ij} / (2N_{ij} + N_i + N_j)$, where N_{ij} is the number of bands shared by both samples, N_i is the number of bands present in i and absent in j, and N_j is the number of bands present in j and absent in i.

Establishment of the core collections

Data of each of the aforementioned four classification approaches were subjected to cluster analysis to divide the entire collection into genetically as distinct groups as possible using SAS version 8.2. (SAS Institute Inc. 1999). Since it was known that there are at least four different morphotypes in the collection (Chapter 4), the resulting dendrograms were truncated at the 4-group level. Random sampling of 10% of the accessions had been shown to capture about 75% of the diversity in the entire collection (Brown 1989b) and was therefore chosen as an adequate size for the core collection. Representative accessions were drawn randomly from each cluster proportional to the number of accessions in that cluster to contribute to the respective core collection (Brown 1989a), until seven or eight accessions (roughly 10% of the entire collection) were assembled. At least one accession was drawn per cluster.

Furthermore, a core collection of seven accessions, drawn from the entire collection by simple random sampling, was used as control to compare the random sampling approach with the approaches based on the information of aforementioned data sets.

Validation of the core collections

The representativeness of each of the four different approaches to establish a core collection ("core quality") on the one hand, and of the randomly assembled control core collection on the other hand, was verified using two different methodologies:

Firstly, the quantitative morphological and agronomic traits of each core collection were compared with the respective reserve collections (= the entire collection minus the respective

core collection) using the Kolmogorov-Smirnov Z test which determines whether two empirical distributions are different. This test is based on the maximum absolute difference *D* between the observed cumulative distribution functions for both samples (Massey Jr. 1951). The cumulative distribution for *n* observations *yi* is defined as $E(x) = \Sigma\, i(yi < x)$, and the test statistics $D\_n^{(+)}=\max(E_1(x)-E_2(x))$ and $D\_n^{(-)}=\max(E_1(x)-E_2(x))$, with E_1 and E_2 being the two empirical distributions. When the difference *D* is significantly large, the two distributions are considered different.

Secondly, the representativeness of the core collections was validated by evaluating the retention of the ranges for morphological and agronomic traits, and of genetic similarity, using the following ratio (Diwan *et al.* 1995): $RR = (\Sigma\, (R_nCC / R_nEC)) / t$, with RR = average range retained, R_nCC = range of variable *n* in the core collection, R_nEC = range of variable *n* in the entire collection, and t = total number of variables compared. All statistical analyses were performed using SPSS version 10.0.7 (SPSS 2000).

Results

Establishing the core collections

For each individual approach (geographic origin, morphological and phenological traits, agronomic and forage quality traits, molecular markers), the characteristics of each cluster, the accessions included and the accessions chosen to enter the core collection are summarized in Table 8.2. At the bottom of the table, the composition of the randomly assembled, i.e. the control core collection, is shown.

Validation of the core collections

a) Comparison of distribution shape

The Kolmogorov-Smirnov Z test showed that the shape of the distribution of morphological and phenological traits was similar in all core collections and their respective reserve collections (Table 8.3). Some differences, however, were detected for the distribution pattern of the agronomic trait DM production and the forage quality trait IVDMD. The distribution shapes of these differed significantly ($P < 0.05$) between the core collections based on morphological and agronomic traits and molecular markers, and their respective reserve collections. On the other hand, the geographic origin approach as well as the control collection did not differ from their respective reserve collections in terms of the distribution of morphological or agronomic traits.

b) Comparison of ranges

When taking a closer look at the ranges, considerable differences became apparent with respect to the extreme – i.e. the minimum and maximum – expressions of traits (Table 8.4).

Table 8.2 Grouping of the *Flemingia macrophylla* collection according to four different characterization approaches, and selection of accessions entering the core collections

Character-ization approach	Cluster No.	Cluster characteristics	Accession (CIAT No.)	*n*	*P*	Accessions in core collection (CIAT No.)
Geographic origin	1	Regions without dry months (i.e. monthly rainfall > 100 mm), accessions of morphotypes M1+M2 mainly from Papua New Guinea and Indonesia	17411, 18437, 19453, 19454, 19457, 19797-19801, 19824, 20616-20618, 20621-20626, 20631, 21529	22	2	19453, 19457
	2	Regions with 4 to 6 dry months (rainfall < 100 mm), accessions of morphotypes M2, M3 and M4 from Hainan/China, Vietnam Central Coast, Malaysia, and Southern and Eastern Thailand	17403-17405, 17407, 17409, 17412, 17413, 18047, 18440, 18438, 20972, 20973, 20975-20980, 20982, 21982, 21990-21993, 21996, 22285	26	2	18440, 21982
	3	Regions with 4 to 6 dry months (rainfall < 100 mm), accessions of all morphotypes from India, Vietnam Northern Highlands, and Central and Northern Thailand	17400, 21079, 21083, 21087, 21090, 21995, 22082, 22087, 22090, 22327, CPI-104890	11	2	17400, 22090
	4	Seven accessions from Africa and tropical America, three accessions without origin information	801, 7184, 20744, 21241, 21248, 21249, 21519, 21580, J-001, I-15146	10	2	7184, 21248
	Total			*69*	*8*	
Morphological and phenological traits	1	Morphotype M1: erect habit, stalked racemes, numerous light pink flowers, black seeds	801, 7184, 20744, 19453, 19454, 19457, 20622, 20625, 20626, 20631, 21241, 21248, 21249, 21519, 21529, 21580, J-001, I-15146, CPI-104890	19	2	21249, 21580
	2	Morphotype M2: semi-erect habit, dark pink flowers in densely congested racemes, glabrous or slightly pubescent stems and petioles	17400, 17403-17405, 17407, 17409, 17411-17413, 18440, 19797-19801, 19824, 20616-20618, 20621, 20623, 20624, 22090	23	2	17404, 17412
	3	Morphotype M3: semi-erect habit, < 20 dark pink or white flowers in very lax racemes which usually do not exceed the length of the petiole	18437, 18438, 21083, 21090, 22082	5	1	21083
	4	Morphotype M4: semi-erect habit, light pink flowers in densely congested racemes, strongly pubescent stems and petioles	18048, 20972, 20973, 20975-20980, 20982, 21079, 21982, 21990-21993, 21995, 21996, 22285, 22327	20	2	18048, 21982
	Total			*67*	*7*	

Agronomic traits and forage quality	1	Intermediate to high DM yield, low IVDMD, high CP content, intermediate regrowth capacity	17400, 17403-17405, 17407, 17409, 17411-17413, 18440, 19457, 19453, 19454, 19797-19801, 19824, 20065, 20616-20618, 20621, 20624	25	2	19801, 19454
	2	High DM yield and CP content, intermediate IVDMD and regrowth capacity	801, 7184, 20744, 20622, 20625, 20626, 20631, 21241, 21248, 21249, 21519, 21529, 21580, J-001, I-15146, CPI-104890	16	2	20626, 20631
	3	High IVDMD and regrowth capacity, intermediate to high DM yield and CP content	18437, 18438, 20975, 21083, 21087, 21090, 22082	7	1	18438
	4	Low to intermediate IVDMD and DM yield, intermediate to high CP content	18048, 20972, 20973, 20976-20980, 20982, 21079, 21982, 21990-21993, 21995, 21996, 22087, 22090, 22285, 22327	21	2	20972, 21079
	Total			*69*	*7*	
Molecular marker (RAPD)	1	*H* = 0.017, *GS* = 0.965	801, 7184, 20744, 19453, 19454, 19457, 20065, 20622, 20625, 20626, 20631, 21241, 21248, 21249, 21519, 21529, 21580, J-001, I-15146, CPI-104890	20	2	20631, 21529
	2	*H* = 0.040, *GS* = 0.918	17400, 17403-17405, 17407, 17409, 17411-17413, 18437, 18440, 19797-19801, 19824, 20616-20618, 20621, 20624	22	2	17407, 20617
	3	*H* = 0.040, *GS* = 0.929	18048, 20972, 20973, 20975-20980, 20982, 21079, 21087, 21982, 21990-21993, 21995, 21996, 22087, 22090, 22285, 22327	23	2	21982, 21996
	4	*H* = 0.140, *GS* = 0.672	18438, 21083, 21090, 22082	4	1	21090
	Total			*69*	*7*	
Control	*Total*		All accessions	*70*	*7*	17400, 17404, 20624, 20626, 21083, 21992, 21580

n, number of accessions of the respective cluster in the total collection; *P*, number of accessions to be chosen as entries for the core collection based on the proportional approach; DM, dry matter; IVDMD, *in vitro* dry matter digestibility; CP, crude protein; *H*, average genetic diversity (Nei 1973); *GS*, Nei and Li genetic similarity (Nei and Li 1979)

Table 8.3 Kolmogorov-Smirnov Z statistics for pairwise comparisons of the distribution of quantitative traits between different *Flemingia macrophylla* core collections and their respective reserve collections

Descriptor	Core collections based on				
	geographic origin	morphological traits	agronomic traits	molecular markers	random sampling (control)
Number of accessions	*n* = 8	*n* = 7	*n* = 7	*n* = 7	*n* = 7
Morphological and phenological traits[a]					
Time to 50% flowering	0.531	0.516	0.593	0.869	0.857
Plant height	0.753	0.468	0.468	0.636	0.769
Leaf area	0.583	0.528	0.569	0.681	0.552
Seed yield	0.471	0.693	0.962	0.792	0.957
1000-seed weight	0.877	0.792	0.630	0.555	0.595
Agronomic and forage quality traits[b]					
DM production (wet season)	0.510	*1.490**	0.699	*1.451**	1.273
DM production (dry season)	0.807	1.131	0.657	*1.523**	1.131
IVDMD (wet season)	1.171	0.748	0.752	0.699	0.433
IVDMD (dry season)	1.148	0.706	*1.594**	0.828	0.776
CP content (wet season)	0.848	0.519	0.821	0.928	0.572
CP content (dry season)	0.883	0.496	0.700	0.533	0.754
Regrowth capacity (wet season)	1.063	1.158	0.819	0.685	0.624

[a] see **Chapter 4**

[b] see **Chapter 5**; DM, dry matter; IVDMD, *in vitro* DM digestibility; CP, crude protein

* values in *italics* indicate significant differences from the reserve collection at $P < 0.05$.

Table 8.4 Ranges of morphological and agronomic descriptors and of genetic similarity for the *Flemingia macrophylla* entire collection and core collections assembled by different approaches, including a randomly sampled control core collection

Descriptor	min-max	Entire collection	Core collections based on*				
			climatic variables	morphological traits	agronomic traits	molecular markers	random sampling (control)
Number of accessions		*n* = 70	*n* = 8	*n* = 7	*n* = 7	*n* = 7	*n* = 7
Morphological and phenological traits[a]							
Time to 50% flowering	180-400	220	84 (38%)	**175 (80%)**	112 (51%)	**175 (80%)**	**166 (75%)**
Plant height	40-287	247	**217 (88%)**	**247 (100%)**	**233 (94%)**	**237 (96%)**	**232 (94%)**
Leaf area	31-216	185	111 (60%)	116 (63%)	95 (51%)	92 (50%)	85 (46%)
Seed yield	3-140	137	99 (72%)	**125 (91%)**	**112 (82%)**	96 (70%)	**136 (99%)**
1000-seed weight	80-243	163	113 (69%)	97 (60%)	87 (53%)	103 (63%)	87 (53%)
Agronomic and forage quality traits[b]							
DM production (wet)	2-753	751	**570 (76%)**	**735 (98%)**	492 (66%)	**720 (96%)**	**624 (83%)**
DM production (dry)	1-391	390	**324 (83%)**	**303 (78%)**	257 (66%)	252 (65%)	**371 (95%)**
IVDMD (wet)	306-581	275	171 (62%)	202 (73%)	**251 (91%)**	**275 (100%)**	202 (73%)
IVDMD (dry)	283-537	254	136 (54%)	168 (66%)	182 (72%)	**190 (75%)**	184 (72%)
CP content (wet)	164-245	81	**72 (89%)**	46 (57%)	46 (57%)	**65 (80%)**	46 (57%)
CP content (dry)	140-252	112	**91 (81%)**	**87 (78%)**	79 (71%)	**93 (83%)**	**104 (93%)**
Regrowth capacity (wet)	2-124	122	54 (44%)	77 (63%)	75 (61%)	86 (70%)	76 (62%)
Molecular marker (RAPD)							
Genetic similarity	0.136-1.000	0.8644	**0.7097 (82%)**	**0.6504 (75%)**	**0.7143 (83%)**	**0.7288 (84%)**	0.6364 (74%)
Range retention (RR)[c]			**69%**	**75%**	**69%**	**78%**	**75%**

[a] see **Chapter 4**

[b] see **Chapter 5**; DM, dry matter; IVDMD, *in vitro* DM digestibility; CP, crude protein

[c] Diwan *et al.* (1995)

***** in ( ) the percentage captured of the diversity in the entire collection; values in **bold** are those ranges capturing at least 75% of the diversity in the entire collection

The core collection based on molecular markers best represented the diversity found in the entire collection, with the ranges of eight out of the thirteen traits capturing at least 75% of the of the entire diversity (average range retention 78%) (Table 8.4). This approach was followed by that based on morphological characteristics and by the control based on random sampling, both with an average range retention of 75%. This agrees with the findings of Brown (1989b) who reported that randomly sampling 10% of a germplasm collection would generally capture about 70% of the diversity in the entire collection. The geographic origin- and the agronomic approaches captured least of the diversity of the entire collection (average range retention 69%), less so than the control collection.

Differences were also detected with respect to the traits concerned: Generally, all approaches captured well the (neutral) genetic similarity in the entire collection (on average ≥ 74%). With respect to agronomic and forage quality traits, however, the core collection based on molecular markers best represented the ranges within the entire collection, with five out of the seven ranges capturing at least 75% of the trait diversity. The core collection based on morphological and phenological characteristics best represented the range, found in the entire collection, of the five morphological traits.

Discussion and conclusions

Comparative validation of the different approaches

Validation of core quality showed that the core collection based on molecular markers best captured the diversity of the entire collection in terms of the ranges of traits, although for two of these the distribution shape differed from that of the entire collection. In general, however, the molecular marker approach apparently captured not only the neutral diversity (genetic similarity), but was also representative in terms of the morphological, agronomic and forage quality variability observed in the entire collection. Molecular marker information has frequently been used for the selection and validation of core collections.

For example, no differences between core and reserve samples for molecular marker data were found for the CIAT common bean core collection (Skroch *et al.* 1998) and for the ICRISAT sorghum core collection (Grenier *et al.* 2000b), which were both established based on classification according to geographic origin, agro-ecological and morphophysiological data (Tohme *et al.* 1995; Grenier *et al.* 2000a).

The morphological approach and the random sampling, i.e. the control approach, were the second best methods for creating core collections, since they captured on average 75% of the diversity in the entire collection and were also representative in the terms of the distribution of traits. The high range retention of the control approach is in agreement with the theoretical estimation of Brown (1989b) who calculated that random sampling of a subset of 10% would

retain approximately 70% of the diversity in the entire collection. Similarly, Malosetti *et al.* (2000) found that the range retention of randomly selected subsets of barley accessions was 80%. Nevertheless, in their study stratified sampling was generally superior to random sampling with an average range retention of 90% in the subsets.

In the present study, the approaches based on agronomic data or geographic origin were insufficient and least representative of the diversity in the entire collection. Generally, information on geographic origin is the approach most commonly used for establishing core collections (Brown and Spillane 1999), due to the fact that geographic origin is the most complete and usually also the most reliable information available in the data bases of germplasm collections. However, knowledge about the relationship between geographic origin and agromorphological and/or genetic diversity is often lacking or insufficient, and differences of this relationship have been observed (Ortiz *et al.* 1998), even between different collections of the same species (Hodgkin *et al.* 1999). In the present work, the lack of relationship confirms findings of previous studies, which have shown that neither morphological (Chapter 4) nor RAPD (Chapter 7) diversity in *F. macrophylla* were correlated with geographic origin. On the contrary, accessions from Africa and tropical America were morphologically and genetically very similar to Asian accessions, and are probably derived from introduced Southeast Asian accessions. Thus, the environments in which some accessions were collected may have been different from the environments in which they originally evolved, leading to a disruption of the relationship between geographic origin and classifications based on morphological traits or genetic markers.

In addition to the accessions that have been selected to enter the core collections, the three accessions CIAT 18437, CIAT 21083 and CIAT 21090 (Chapter 5) selected for further evaluation based on agronomic and forage quality evaluations, as well as control accession CIAT 17403, frequently included in other studies, should also be included in order to ensure the presence of (regionally) promising accessions within the core.

Different strategies for creating core collections have been investigated, and the theoretical properties of many of them are known (glycine, Brown 1989a; Peeters *et al.* 1990; sorghum, Noirot *et al.* 1996; sorghum, Grenier *et al.* 2001; sugarcane, Nair and Balakrishnan 2003). Nevertheless, applying the same strategy to collections of different species may give contrasting results due to differences in (i) collection patterns, (ii) objectives and priorities of germplasm curators, and (iii) reproductive biology and genetic structure of species (Skroch *et al.* 1998). Based on the hypothesis that any conclusions will be more accurate and more sensible if data are analysed in as many ways as possible, it would be interesting to validate an approach based on the combination of different data sets (geographic origin, morphological, agronomic, genetic information) to create core collections. The combination of different diversity estimates might result in a more precise estimate of genetic relationship

and thus in more representative core collections than approaches based on individual data sets. Different combinations of data sets could then be compared to assess the most efficient strategy to create core collections.

Of the two methods for validating core quality, the analysis of the distribution shapes was less informative and hardly detected any differences between the different core collection approaches. The comparison of ranges was more sensible. At the same time, the latter method is particularly informative for the plant breeder who is usually mainly interested in extreme expressions of particular traits (Holbrook *et al.* 1993). Based on the results of this study, the comparison of ranges thus seems to be a more useful strategy to evaluate core quality than the comparison of distribution shapes. However, these findings should be tested with more traits to verify if this is generally the case. Furthermore, the core collections based on morphological and agronomic information should be regionally tested in multilocational trials to verify if the results obtained in different environments are in agreement with those in the present work. Finally, similar studies are needed to test the applicability of these results to other wild legume species.

Practical implications

The practical implications of the results have to be analysed carefully, taking into consideration the particular case of the relatively small germplasm collections of perennial wild tropical legumes. The assessment of genetic diversity with molecular markers can indeed constitute a powerful tool for the establishment of core collections. However, certain pre-conditions need to be met. Even if markers are used that do not require any prior sequence information (e.g. RAPD or AFLP), laboratory analysis can usually only be concluded within several months – if no problems occur and if the methodology is already established.

This is, however, usually not the case with wild species such as most MPT legumes, and the establishment of appropriate DNA extraction protocols and amplification conditions can require considerable time and resource inputs. The justification of the (time and cost) input needs to be analysed even more carefully with view to the generally small size of collections, which may render the costly establishment of complicated laboratory procedures such as AFLP inefficient. Although the RAPD technology is increasingly being replaced by methods with better reproducibility (e.g. AFLP, Jones *et al.* 1997a; Savelkoul *et al.* 1999), it might still be recommendable for the analysis of small wild legume collections due to the ease of implementation.

However, it has to be kept in mind that the diversity captured by molecular markers is only a relative measure of similarity (or dissimilarity) between accession-pairs and provides little information directly relevant for plant improvement. For example, molecular analysis shows

that accession *x* is highly different from accession *y*, but it provides little information as to *how* in terms of morphological, agronomic or forage quality traits. Nevertheless, as a pre-screening technique to select a core collection representative of the entire collection in terms of genetic diversity, it can reduce the number of accessions for more intensive conventional characterization techniques.

Morphological characterization could be an effective alternative as sampling strategy for the creation of core collections (Prasada Rao and Rao 1995; Noirot *et al.* 1996; Upadhyaya *et al.* 2003). It is one of the standard germplasm management procedures and provides important basic information about (morphological) diversity in germplasm collections, including the detection of possible infraspecific taxa. For example, in the *F. macrophylla* collection, four different morphotypes were identified that have taxonomic implications (Chapter 4), and greatly determined the pattern of agronomic (Chapter 5) and forage quality traits (Chapter 6) and molecular markers (Chapter 7) detected in the collection. Furthermore, even if a field plot has been established for a single approach originally, it can be used in multiple ways. For example, a plot for morphological and phenological characterization could also be used for the assessment of seed production and other agronomic traits, and vice versa.

The selection of accessions based on geographic origin alone is a cost-efficient, fast strategy for the establishment of core collections. However, its representativeness in terms of diversity of other traits is variable, and in the present study the approach resulted in a core collection which was even less representative than that based on random sampling. For other species or in combination with other approaches, however, sampling based on information of geographic origin might be a useful strategy.

Finally, the results showed that core collections could also be established by random sampling, avoiding costs and time and still obtaining a high degree of representativeness regarding traits of major interest. In the absence of funds, random sampling is thus a valuable and efficient strategy for the creation of core collections. It has been shown that this approach not only preserves a high percentage of the total diversity in the collection in terms of means and variances (Brown 1989b), but also has a high range retention.

Nevertheless, the following needs to be kept in mind: If a core collection is assembled based on the grouping of accessions according to previously available data (e.g. morphological data), then it is possible to trace back, for each accession in the core collection, to which group of accessions with similar characteristics it belongs in the entire collection. Thus, if an accession in the core collection is identified with a particular, new trait (e.g. disease resistance), then the respective group of (morphologically) similar accessions in the reserve collection can be identified. Further evaluations can then focus on the group of similar accessions in the reserve collection to identify more promising accessions (Miklas *et al.*

1999), allowing a more efficient use of time and cost resources. This approach, however, is not viable in the case of randomly assembled core collections, since the accessions forming the core are not linked to the remaining accessions in the reserve collection. Decisions regarding the most appropriate core collection strategy therefore need to be taken on a case-by-case basis, analysing carefully whether priority should be given to higher core quality (= high representativeness) or to greater resource efficiency.

Chapter 9

Summary

Cratylia argentea (Desv.) O. Kuntze and *Flemingia macrophylla* (Willd.) Merrill are promising tropical multipurpose shrub legumes. Both are drought-tolerant, well adapted to low-fertility, acid soils, and especially suited for low-input smallholder production systems in the sub-humid and humid tropics. They can be used in multiple ways, for example as dry season forage supplementation, live soil cover or mulch, erosion barrier hedges, and shade-providing shrubs in young coffee and cocoa plantations. Fairly comprehensive germplasm collections have been assembled from the wild-legume flora in Brazil (*C. argentea*) and Southeast Asia (*F. macrophylla*), but research and development are so far based on only a few accessions and knowledge about the extent of genetic diversity within these collections is very limited. In addition, the potential utilization of *F. macrophylla* is so far limited by poor forage quality and acceptability of the few evaluated accessions.

The objective of the present study, conducted in a research cooperation with the International Centre for Tropical Agriculture (CIAT), Cali, Colombia, was to assess the diversity in the germplasm collections of *C. argentea* (38 accessions) and *F. macrophylla* (69 accessions) in terms of morphological and phenological traits, agronomic and forage quality traits, and molecular markers, and to identify superior genotyes. Based on these different characterization approaches, the objective was furthermore to establish core collections for *F. macrophylla*, and to compare and validate the different strategies, giving particular consideration to their practical implications (time and cost efficiency) for the application to small collections of perennial wild tropical legumes.

Cratylia argentea

In the case of *C. argentea*, high diversity in terms of phenological and agronomic as well as forage quality traits (flowering, seed yield, regrowth capacity, dry matter (DM) production, *in vitro* dry matter digestibility (IVDMD) and crude protein (CP) content) was detected in the collection, with scope for plant improvement in terms of higher dry season DM production. The accessions CIAT 18674 and CIAT 22406 were identified as promising for further evaluation since they were similar to the commercial cultivar “Veraniega” in terms of forage quality, and superior in terms of DM production, particularly in the dry season.

Molecular marker analysis with random amplified polymorphic DNA (RAPD) showed that the genetic diversity in the *C. argentea* collection was relatively low and fairly homogeneously distributed. The accessions CIAT 22373, CIAT 22378, CIAT 22380, CIAT 22381, and CIAT 22411 were identified as possible duplicates, and bulking them into a

single accession is suggested since they were also very similar morphologically and agronomically.

Molecular marker evidence suggested that outcrossing has occurred and is still occurring among *C. argentea* accessions and precautions should be taken by genebanks to avoid cross-pollination during seed multiplication in the field until more information about the reproduction system and outcrossing rates of *C. argentea* is available. Multiplication protocols should be re-considered to maintain the genetic integrity of accessions. Furthermore, the genetic diversity of accessions repeatedly multiplied in the genebank should be compared with that of the respective "original" accessions to determine whether outcrossing has occurred during or before *ex situ* storage. Based on this information, it should be decided whether the maintenance of individual accessions is desirable, or whether they should be pooled.

Flemingia macrophylla

For *F. macrophylla*, high diversity in terms of morphological and agronomic as well as forage quality traits (plant height, inflorescence and seed characteristics, regrowth capacity, DM production, IVDMD and tannin content) was detected among the 69 accessions. The identification of four morphotypes in the collection probably has taxonomic implications. Scope for plant improvement was identified with respect to forage quality – one of the species' main limitations. The accessions CIAT 18437, CIAT 21083 and CIAT 21090 had similar DM production and higher digestibility than the control accession, and were virtually free of extractable condensed tannins. Problems with low palatability and low seed production of these promising accessions need to be further studied.

Overall genetic diversity in the *F. macrophylla* collection was higher than in *C. argentea*, and its distribution pattern corresponded closely to the four morphotypes revealed by conventional characterization. Various duplicate accessions were identified, and evidence was provided that the non-Asian *F. macrophylla* accessions are not native to their collection site regions, but rather introduced from Southeast Asia.

The comparison of different strategies to create core collections using *F. macrophylla* as example, showed that core collections based on molecular markers best captured the diversity in the entire collection, followed by those based on morphological data and random sampling. Using the latter strategy, 75% of the ranges of traits were retained, making random sampling a viable and resource-efficient option for creating core collections in the absence of other data or when time and funds are restricted. Decisions should be taken on a case-by-case basis to determine whether priority is given to higher core quality (= higher representativeness) or to

greater resource efficiency. Similar studies are needed to test the applicability of these results to other wild legume species.

Overall, the results have direct applications for plant improvement of these promising multipurpose legumes. The superior genotypes selected in this study will be used in work with farmers in CIAT-research sites in Central America and distributed to partners. It must be recognized, however, that the diversity assessed is influenced by the climatic and edaphic conditions at the site where the studies were conducted. Therefore, multilocational trials should be considered with a selected subset (including the promising accessions) of *C. argentea* and *F. macrophylla* i) to assess the extent of genotype x environment interaction, and ii) to identify genotypes with consistently high performance in a range of distinct environments. Furthermore, research on the reproduction system of both species is urgently required to determine the potential extent and impact of outcrossing.

Beyond the immediate application of these species for farmer utilization, the results of the use and comparison of different approaches to assess diversity and to establish core collections can help to improve germplasm management and characterization of wild tropical legume species in general. Random sampling has been identified as a valuable and resource-efficient strategy for the creation of core collections when no additional information about accessions is available, and in the absence of adequate funds. The validation of the findings of this study with a broader range of perennial tropical wild legumes is necessary to assess their applicability to other species.

Chapter 10

Zusammenfassung

Die tropischen Mehrzweck-Buschleguminosen *Cratylia argentea* (Desv.) O. Kuntze und *Flemingia macrophylla* (Willd.) Merrill werden wegen ihrer Trockentoleranz und Anpassung an nährstoffarme, saure Böden als vielversprechend angesehen. Deren Anbau ist besonders für Kleinbauern geeignet, und sie können in vielfältiger Weise eingesetzt werden, z.B. zur Futterergänzung während ausgedehnter Trockenzeiten, als Bodendecker, Mulch und Lebendzäune, zum Erosionsschutz sowie als Schattenbäume. Umfassende Samenkollektionen sind verfügbar, mit Akzessionen von Wildpopulationen aus Brasilien (*C. argentea*) und Südostasien (*F. macrophylla*). Die Erforschung und Verbesserung beider Arten beschränkte sich bislang lediglich auf wenige Akzessionen, und das Ausmaß der genetischen Diversität innerhalb der Sammlungen ist daher praktisch unbekannt. Zudem ist das Potenzial der bisher bekannten Akzessionen von *F. macrophylla* durch einen relativ niedrigen Futterwert limitiert.

Das Ziel der vorliegenden Arbeit war im Rahmen einer Forschungskooperation mit dem Centro Internacional de Agricultura Tropical (CIAT), Cali, Kolumbien, die verfügbare genetische Diversität von *C. argentea* (38 Akzessionen) und *F. macrophylla* (69 Akzessionen) anhand morphologischer und phänologischer Merkmale, agronomischer und Futterwert-Eigenschaften, sowie anhand molekularer Marker zu erfassen und zu beschreiben, und vielversprechende Akzessionen zu identifizieren. Weiterhin sollten beispielhaft anhand von *F. macrophylla* verschiedene Strategien zur Erstellung von *core collections* – basierend auf den o.g. Ansätzen zur Erfassung der Diversität – verglichen und validiert werden, und deren praktische Bedeutung (Kosten, Zeitaufwand) für die Erstellung von *core collections* mehrjähriger tropischer Wildleguminosen im allgemeinen untersucht werden.

Cratylia argentea

In der *C. argentea* Kollektion wurde eine grosse Diversität ermittelt (Tage bis Blühbeginn, Samenproduktion, Wiederaustrieb, Trockenmasseproduktion, *in vitro* Verdaulichkeit der Trockenmasse (IVDMD), Rohprotein-Gehalt), sowie Potenzial zur Sortenverbesserung in Bezug auf höhere Trockenmasseproduktion, besonders in der Trockenzeit. Die Akzessionen CIAT 18674 und CIAT 22406, die eine ähnlich hohe Futterqualität wie die Sorte „Veraniega“ aufwiesen, wurden wegen ihrer höheren Trockenmasseproduktion für weitere Evaluierungen ausgewählt.

Die molekulare Analyse mit „random amplified polymorphic DNA“ (RAPD)-Markern zeigte, dass die genetische Diversität in der *C. argentea* Kollektion relativ gering war. Die

Akzessionen CIAT 22373, CIAT 22378, CIAT 22380, CIAT 22381 und CIAT 22411 wurden als mögliche genetische Duplikate identifiziert. Es wird daher vorgeschlagen, sie zu einer einzigen Akzession zusammen zu fassen, da sie sich auch in Bezug auf morphologische und agronomische Merkmale sehr ähnlich waren.

Weiterhin lassen die Ergebnisse der molekularen Marker Analyse auf das Vorkommen von Auskreuzungen schließen. Den Genbanken wird deshalb empfohlen, die Vorgehensweise bei der Vermehrung von *C. argentea* zu überprüfen und gegebenenfalls Maßnahmen zu ergreifen, um die Kreuzbestäubung zwischen Akzessionen zu verhindern. Außerdem sollte die genetische Diversität der durch die Genbank vermehrten Akzessionen mit der Diversität der entsprechenden Originalakzessionen verglichen werden, um zu ermitteln, ob Auskreuzungen bereits in den natürlichen Populationen der Wildflora oder erst in der Genbank stattgefunden haben. Anhand dieser Information sollte dann entschieden werden, ob sämtliche Akzessionen individuell erhalten werden sollten, oder ob einige zusammengefasst werden können.

Flemingia macrophylla

In der *F. macrophylla* Kollektion wurde eine große Diversität für mehrere Eigenschaften (Pflanzenhöhe, Blüten- und Fruchtmerkmale, Wiederaustrieb, Trockenmasseproduktion und IVDMD) ermittelt. Eine Beschreibung von vier unterschiedlichen Morphotypen hat wahrscheinlich taxonomische Bedeutung. Sortenverbesserung im Hinblick auf höhere Futterwerte – einem der das Potenzial der Art limitierenden Faktoren – scheint aussichtsreich zu sein. Die Akzessionen CIAT 18437, CIAT 21083 and CIAT 21090 wiesen eine ähnliche Trockenmasseproduktion wie die als Kontrolle dienende Akzession auf, aber höhere IVDMD sowie praktisch keine Tannin-Gehalte. Schmackhaftigkeitsprobleme und die geringe Samenproduktion dieser Akzessionen erfordern weitere Aufmerksamkeit.

Die genetische Diversität in der *F. macrophylla* Kollektion war größer als die in *C. argentea*, und ihre Struktur entsprach weitestgehend der Diversität von morphologischen Merkmalen. Es wurden mehrere genetische Duplikate identifiziert. Die Ergebnisse deuten darauf hin, dass die nicht-asiatischen *F. macrophylla* Akzessionen nicht nativ an ihren Sammelorten vorkommen, sondern wahrscheinlich von aus Südostasien eingeführten Akzessionen abstammen.

Der Vergleich der verschiedenen *core collection*-Strategien zeigt, dass die Erstellung von *core collections* anhand molekularer Marker die Diversität der Gesamtkollektion am besten widerspiegelte, gefolgt von der Strategie basierend auf morphologischen Merkmalen und anhand einer zufälligen Auswahl der Akzessionen. Letztere erfasste 75% der Variationsbreite wichtiger Merkmale und stellt daher eine zuverlässige und ökonomische Alternative zur *core collection* Erstellung dar, sofern keine anderen Informationen über die Akzessionen

vorhanden sind, oder wenn finanzielle Ressourcen und Zeit limitierende Faktoren darstellen. Die Entscheidung für eine bestimmte Strategie sollte von Fall zu Fall getroffen werden, in Abhängigkeit davon, ob höherer *core*-Qualität (d.h. höhere Repräsentativität) oder höherer Ressourcen-Effizienz der Vorzug gegeben wird. Ferner sollte die Anwendbarkeit der Ergebnisse auf andere Arten überprüft werden.

Die Ergebnisse der vorliegenden Arbeit werden insofern direkt angewandt, indem die als besonders geeignet identifizierten Akzessionen in on-farm Versuchen des CIAT und seiner Partner in Mittelamerika weiter getestet werden. Es muss jedoch berücksichtigt werden, dass die in der vorliegenden Arbeit ermittelte Diversität von den klimatischen und edaphischen Bedingungen am Evaluierungsstandort beeinflusst ist. Die Durchführung multi-lokationaler Versuche mit ausgewählten *subsets* (einschließlich der vielversprechendsten Akzessionen) von *C. argentea* and *F. macrophylla* ist deshalb erforderlich, um i) das Ausmaß der Genotyp x Umwelt Interaktion zu erfassen, sowie ii) Genotypen mit durchgängig guten Eigenschaften unter verschiedenen Umweltbedingungen zu identifizieren. Weiterhin wird die Notwendigkeit der Bestimmung der Reproduktionsstrategie beider Arten hervorgehoben, um das Ausmaß der Auskreuzungen zu ermitteln.

Abgesehen von der unmittelbaren Anwendbarkeit dieser beiden Mehrzweck-Arten durch Kleinbauern können die Ergebnisse der Vergleiche der unterschiedlichen Ansätze zur Ermittlung der Diversität und zur Erstellung von *core collections* dazu beitragen, das Management und die Evaluierung von tropischen Wildleguminosen allgemein zu verbessern. Die Zufalls-Strategie wurde als zuverlässige und Ressourcen sparende Alternative zur *core collection* Erstellung identifiziert, wenn fehlende Informationen oder knappe Ressourcen limitierende Faktoren sind. Die Ergebnisse sollten anhand anderer tropischer Wildleguminosen auf ihre Übertragbarkeit auf weitere Arten überprüft werden.

Chapter 11

Resumen

Cratylia argentea (Desv.) O. Kuntze y *Flemingia macrophylla* (Willd.) Merrill son prometedoras leguminosas tropicales arbustivas multipropósito. Ambas son tolerantes a la sequía y adaptadas a suelos ácidos e infértiles. Son particularmente útiles para pequeños productores en los trópicos sub-húmedos y húmedos. Se pueden usar de múltiples formas, por ejemplo como suplemento alimenticio para ganado en la época seca, como cobertura o mulch, como setos vivos para evitar la erosión, y para sombrío en plantaciones jóvenes de café y cacao. Las colecciones existentes compiladas de la flora silvestre de Brasil (*C. argentea*) y del Sudeste de Asia (*F. macrophylla*) son relativamente extensas. Sin embargo, hasta ahora la investigación y el desarrollo de estas especies han sido basados solamente en pocas accesiones, y por ende el conocimiento acerca de la diversidad genética en las colecciones es muy limitado. Además, en este momento el potencial de utilización de *F. macrophylla* está limitado debido a la baja calidad nutritiva y baja palatabilidad de las pocas accesiones evaluadas.

El objetivo del presente trabajo que se condujo en colaboración con el Centro Internacional de Agricultura Tropical (CIAT), Cali, Colombia, fue determinar la diversidad en las colecciones de germoplasma de *C. argentea* (38 accesiones) y *F. macrophylla* (69 accesiones) en términos de características morfológicas, fenológicas, agronómicas, de calidad nutritiva y de marcadores moleculares, e identificar genotipos con desempeño superior. Basado en los diferentes enfoques, el objetivo fue además establecer colecciones núcleo (*'core collections'*) de *F. macrophylla* y comparar y validar las diferentes estrategias, con énfasis en sus implicaciones prácticas (eficiencia en términos de tiempo y costos) y su aplicabilidad a colecciones de otras leguminosas tropicales silvestres y perennes.

Cratylia argentea

En el caso de *C. argentea*, se detectó una gran diversidad en la colección en términos de características fenológicas, agronómicas y de calidad nutritiva (floración, producción de semilla, capacidad de rebrote, producción de materia seca, digestibilidad *in vitro* de la materia seca y contenido de proteína cruda), con potencial especial para el mejoramiento en términos de mayor producción de materia seca en la época seca. Las accesiones CIAT 18674 y CIAT 22406 fueron seleccionadas para posteriores evaluaciones más extensas, basado en su similitud al cultivar comercial "Veraniega" en términos de calidad nutritiva, y su superioridad en cuanto a producción de materia seca, particularmente en la época de sequía.

El análisis de marcadores moleculares con RAPDs (*"random amplified polymorphic DNA"*) mostró que la diversidad genética en la colección de *C. argentea* era relativamente baja y su distribución muy homogénea. Las accesiones CIAT 22373, CIAT 22378, CIAT 22380, CIAT 22381 y CIAT 22411 fueron identificadas como posibles duplicados. Debido además a su similitud morfológica y agronómica, se recomienda reunirlas en una sola accesión.

Evidencia molecular sugiere la probabilidad de cruzamiento entre accesiones de *C. argentea*. Se recomienda que los bancos de germoplasma tomen medidas para evitar la polinización cruzada durante la multiplicación de semillas hasta que mayor información esté disponible acerca del sistema reproductivo y la dimensión de polinización cruzada en *C. argentea*. Los procedimientos de multiplicación se deberían reconsiderar para mantener la integridad genética de las accesiones. Además, se debería comparar la diversidad genética de las accesiones multiplicadas en el banco de germoplasma con la de las accesiones "originales" para determinar si el cruzamiento ocurrió entre las poblaciones naturales de la flora silvestre o en el banco de germoplasma. Basado en esta información, se debería decidir si se desea mantener todas las accesiones individuales o si algunas se deberían unir.

Flemingia macrophylla

Entre las 69 accesiones de la colección de *F. macrophylla* se detectó una gran diversidad en cuanto a características morfológicas, agronómicas y de calidad nutritiva (altura de las plantas, características de la inflorescencia y del fruto, capacidad de rebrote, producción de materia seca, digestibilidad *in vitro* y contenido de taninos). La identificación de cuatro morfotipos en la colección probablemente tiene implicaciones taxonómicas. Se descubrió potencial de mejoramiento en cuanto a calidad nutritiva – una de las limitaciones principales de la especie. Las accesiones CIAT 18437, CIAT 21083 y CIAT 21090 tenían una producción de materia seca similar al control pero mayor digestibilidad *in vitro*, y eran virtualmente libres de taninos condensados solubles. La baja palatabilidad y la baja producción de semillas de estas accesiones prometedoras son problemáticas y necesitan ser estudiadas más a fondo.

En general, la diversidad genética en la colección de *F. macrophylla* fue mayor que en *C. argentea*, y el patrón de distribución geográfica correspondió bien a los cuatro morfotipos revelado por la caracterización convencional. Varios duplicados fueron identificados, y hubo evidencia de que las accesiones no-asiáticas de *F. macrophylla* no son nativas de las regiones de su recolección sino introducidas del Sudeste de Asia.

La comparación de las diferentes estrategias para crear colecciones núcleo, usando *F. macrophylla* como ejemplo, mostró que las colecciones núcleo basadas en marcadores moleculares capturaron mejor la diversidad que existe en la colección entera, seguido por aquellas basadas en información morfológica y compiladas al azar. Usando la última

estrategia, se conservó el 75% de la diversidad, lo que convierte la compilación al azar en una opción viable y eficiente para la creación de colecciones núcleo si no existe otra información o si el tiempo o los recursos económicos son factores limitantes. Decisiones acerca de la estrategia más apropiada deberían ser tomadas según el caso, analizando si se da prioridad a más calidad (= mayor representatividad) de la colección núcleo o a mayor eficiencia con respecto a los recursos. Se requiere de estudios similares para evaluar la transferibilidad de las estrategias a otras especies de leguminosas silvestres.

En general, los resultados tienen aplicaciones directas para el mejoramiento de estas prometedoras leguminosas multipropósito. Los genotipos con desempeño superior seleccionados en este trabajo van a ser utilizados en estudios con pequeños productores en Centroamérica y van a ser distribuidos a instituciones colaboradoras. Sin embargo, hay que mencionar que la diversidad determinada en este trabajo es afectada por las condiciones climáticas y edáficas del lugar donde se realizaron los ensayos. Por ende, se requiere de ensayos multilocacionales con una selección de accesiones (incluyendo las accesiones prometedoras) de *C. argentea* y *F. macrophylla* con el fin de identificar i) interacciones genotipo con el medio ambiente, y ii) genotipos con consistente desempeño superior en localidades contrastantes. Además, se requiere urgentemente de estudios acerca del sistema de reproducción de ambas especies para determinar la dimensión y el impacto de polinización cruzada.

Más allá de la aplicación inmediata de estas especies para la utilización a través de pequeños productores, los resultados de la comparación de diferentes estrategias para determinar la diversidad y para crear colecciones núcleo pueden ayudar a mejorar el manejo del germoplasma y la caracterización de leguminosas tropicales silvestres en general. La compilación al azar fue identificada como una estrategia valiosa y eficiente para la formación de colecciones núcleo en caso de falta de información adicional acerca de las accesiones o de fondos adecuados. Sin embargo, ensayos similares son necesarios con un rango más amplio de especies leguminosas tropicales silvestres y perennes para validar los resultados de este trabajo y para determinar su transferibilidad a otras especies.

Chapter 12

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List of abbreviations

ADF	acid detergent fibre
ANOVA	analysis of variance
bCT	bound condensed tannins
BSA	bovine serum albumin
CGIAR	Consultative Group on International Agricultural Research
CIAT	International Centre of Agriculture, Cali, Colombia
CP	crude protein
CT	condensed tannins
DM	dry matter
DNA	deoxyribonucleic acid
EMBRAPA	Empresa Brasileira de Pesquisa Agropecuária, Brazil
FAO	Food and Agriculture Organization of the United Nations, Rome, Italy
GIS	Geographic Information Systems
GLM	general linear model
GS	genetic similarity
H	heterogeneity (= genetic diversity)
HPLC	high-performance liquid chromatography
IBPGR	International Board for Plant Genetic Resources; now IPGRI
ILDIS	International Legume Database and Information Service
IPGRI	International Plant Genetic Resources Institute, Rome, Italy
IVDMD	*in vitro* dry matter digestibility
LSD	least significant difference
MCA	multiple correspondence analysis
MPT	multipurpose shrub and tree legumes
N	nitrogen
n	number of observations
n.a.	not available
ND	not detectable
ns	not significant
N-ADF	acid detergent fibre-bound nitrogen
NDF	neutral detergent fibre
P	probability
PBC	protein-binding capacity
PBE	protein-binding entities
PCA	principal component analysis
PCR	polymerase chain reaction

PGR	plant genetic resources
r	correlation coefficient
RAPD	random amplified polymorphic DNA
RPI	relative palatability index
sCT	soluble condensed tannins
SEM	standard error of the mean
SINGER	System-wide Information Network for Genetic Resources of the CGIAR
TE	Tris-HCl EDTA buffer
UNCED	United Nations Conference on Environment and Development
UPGMA	unweighted pair group method with arithmetic averages
UV/VIS	ultra violet/visible
v/v	volume/volume
w/w	weight/weight

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