Diversity studies in the interaction between the anthracnose fungus *Colletotrichum gloeosporioides* and its host plant *Stylosanthes* spp. in Mexico

S. Gama-López, F. Munaut, J. Vander Stappen, X. Scheldeman and V. Van Damme

IPGRI and INIBAP
operate under the name
Bioversity International

Supported by the CGIAR

Diversity studies in the interaction between the anthracnose fungus *Colletotrichum gloeosporioides* and its host plant *Stylosanthes* spp. in Mexico

S. Gama-López[1], F. Munaut[2], J. Vander Stappen[3], X. Scheldeman[4] and V. Van Damme[5]

[1] Jardín Botánico del Instituto de Biología, Universidad Nacional Autónoma de México, México D.F. 04510, México
[2] Université catholique de Louvain, Faculté d'Ingénierie biologique, agronomique et environnementale, Unité de Microbiologie, Mycothèque de l'UCL (BCCM/MUCL), Louvain-la-Neuve, Belgium
[3] Katholieke Universiteit Leuven, Faculty of Bioscience Engineering, Laboratory of Gene Technology, Leuven, Belgium
[4] Bioversity International, Understanding and Managing Biodiversity Programme, Cali, Colombia
[5] Consultant, Cali, Colombia

Bioversity International is an independent international scientific organization that seeks to improve the well-being of present and future generations of people by enhancing conservation and the deployment of agricultural biodiversity on farms and in forests. It is one of 15 centres supported by the Consultative Group on International Agricultural Research (CGIAR), an association of public and private members who support efforts to mobilize cutting-edge science to reduce hunger and poverty, improve human nutrition and health, and protect the environment. Bioversity has its headquarters in Maccarese, near Rome, Italy, with offices in more than 20 other countries worldwide. The Institute operates through four programmes: Diversity for Livelihoods, Understanding and Managing Biodiversity, Global Partnerships, and Commodities for Livelihoods.

The international status of Bioversity is conferred under an Establishment Agreement which, by January 2007, had been signed by the Governments of Algeria, Australia, Belgium, Benin, Bolivia, Brazil, Burkina Faso, Cameroon, Chile, China, Congo, Costa Rica, Côte d'Ivoire, Cyprus, Czech Republic, Denmark, Ecuador, Egypt, Greece, Guinea, Hungary, India, Indonesia, Iran, Israel, Italy, Jordan, Kenya, Malaysia, Mali, Mauritania, Morocco, Norway, Pakistan, Panama, Peru, Poland, Portugal, Romania, Russia, Senegal, Slovakia, Sudan, Switzerland, Syria, Tunisia, Turkey, Uganda and Ukraine.

Financial support for Bioversity's research is provided by more than 150 donors, including governments, private foundations and international organizations. For details of donors and research activities please see Bioversity's Annual Reports, which are available in printed form on request from bioversity-publications@cgiar.org or from Bioversity's Web site (www.bioversityinternational.org).

Citation: Gama-López S, Munaut F, Vander Stappen J, Scheldeman X, Van Damme V. 2007. Diversity studies in the interaction between the anthracnose fungus *Colletotrichum gloeosporioides* and its host plant *Stylosanthes* spp. in Mexico. Technical report of Bioversity International, Rome, Italy. x + 82 p.

ISBN: 978-92-9043-769-7

Bioversity International
Via dei Tre Denari, 472/a
00057 Maccarese
Rome, Italy

Contents

List of tables

List of figures

List of maps

Acronyms used in the text

ACIAR	Australian Centre for International Agricultural Research
AFLP	amplified fragment length polymorphism
ANN	artificial neural network
asi	anthracnose severity index
CIAT	International Centre for Tropical Agriculture
CSIRO	Commonwealth Scientific and Industrial Research Organisation (Australia)
DGDC	Directorate-General for Development Cooperation (Belgium)
EMBL	European Molecular Biology Laboratory
IGS	intergenic spacer region
ILCA	International Livestock Centre for Africa
IPGRI	International Plant Genetic Resources Institute, *now* Bioversity International
ITS	internal transcribed spacer
KUL	Katholieke Universiteit Leuven (Belgium)
LM	light microscope
PCA	principal component analysis
PCR	polymerase chain reaction
PDA	potato dextrose agar
RAPD	randomly amplified polymorphic DNA
RFLP	restriction fragment length polymorphism
SEM	scanning electron microscope
SSR	single-sequence repeat
STS	sequence-tagged sites
UCL	l'Université catholique de Louvain (Belgium)
UNAM	Universidad Nacional Autónoma de México
UPGMA	unweighted pair group method with arithmetic averages

Foreword

Pests and diseases are important constraints to production in both traditional and modern agricultural systems. It is widely accepted that crop diversity, mainly through use of resistance and tolerance genes, is an important asset in reducing the risk of crop losses related to pests and diseases. However, little is known about the effect of the natural pathogen diversity on the occurrence and severity of phytopathological infestations.

This publication summarizes the results of the multidisciplinary project 'Genetic diversity studies in the interaction between the anthracnose fungus *Colletotrichum gloeosporioides* and its host plant *Stylosanthes* spp.' The legume *Stylosanthes* is an important forage crop worldwide and *Colletotrichum gloeosporioides* is its most important pathogen. This project was a multidisciplinary bi-national effort centred in Mexico, a centre of origin of the host plant, which focused on characterizing both the host plant and the pathogen using different characterization techniques, from macro-morphological through molecular.

As anthracnosis is reducing *Stylosanthes* yields from Africa to Australia, an increased knowledge and understanding of the co-existence of crop and pathogen diversity will benefit stakeholders outside the study area as well. A team of international researchers undertook a coordinated effort to increase the inclusion of information on host and pathogen diversity in areas where the crop and its pathogen are native. The Unité de Phytopathologie de l'Université catholique de Louvain, Louvain-la-Neuve, Belgium (UCL) focused on the characterization of *C. gloeosporioides* and other *Colletotrichum* species associated with wild *Stylosanthes* species in Mexico, while *Stylosanthes* diversity and taxonomy were studied by the Laboratorio de Recursos Naturales, Unidad de Biología, Tecnología y Prototipos, Facultad de Estudios Superiores Iztacala de la Universidad Nacional Autónoma de México (UNAM) at the morphological level and by the Laboratory of Gene Technology, Katholieke Universiteit Leuven, Belgium (KUL) that studied the material at the molecular level. The Mexican partner, UNAM, was responsible for the collection of materials, both host plant and pathogen, while the Belgian partners, UCL and KUL, carried out the molecular analysis. This study is a clear example of how a collaborative, multidisciplinary effort, including the exchange of plant material, allows for the optimal use of existing synergies between different research centres, leading to a better understanding of a complex theme such as host-pathogen diversity. This will permit a better use of the crop's genetic diversity, and the corresponding resistance genes available, as well as the application of better screening methods for pest or disease resistance, based on a more extensive pathogen diversity.

Bioversity International, formerly known as IPGRI, and its Regional Office for the Americas in Cali, Colombia is honoured that it was allowed to coordinate this project.

Acknowledgements

Special thanks go to Emile Frison and Daniel Debouck, who were the initiators of this multidisciplinary research project, and to their successor, Luigi Guarino, who has been the project coordinator and who offered his expertise on the conservation of *Stylosanthes* species in Mexico. The guidance of the local scientific supervisors and professors Guido Volckaert, Henri Maraite and Patricia Dávila during the whole project was highly appreciated. The authors Munaut and Vander Stappen want to acknowledge explicitly the technical assistance of respectively Nancy Hamaide and Ingrid Weltjens during the whole research project. Author Gama-López would like to thank the curators of the herbaria of BM, BR, ENCB, F, GH, IBUG, K, MEXU, MO, NY and US for facilitating the use of specimens for this study.

We must acknowledge the linguistic and technical support of Marleni Ramirez, Carmen de Vicente and Segenet Kelemu, who in spite of their very tight working schedule were so kind as to collaborate with this publication. Thanks also to Wendy Hernández Rodríguez, the scientific illustrator for elaborating the botanical drawing for Figure 7. A special thank you to Michael Hermann and Veerle Van den Eynden, who were always at our disposal to resolve translation problems with specialized technical terms. Final editing and preparation for publication on behalf of Bioversity International was by Thorgeir Lawrence.

Finally, many thanks to the Belgian Directorate-General for Development Cooperation (DGDC), who funded the project and made the publication of this report possible.

1. Background to the project

The project 'Genetic diversity studies in the interaction between the anthracnose fungus *Colletotrichum gloeosporioides* and its host plant *Stylosanthes* spp.' was implemented in two phases, from 1993 to 1995 and from 1997 to 1999, with the support of the Belgian Directorate-General for Development Cooperation (DGDC). The project was undertaken by Bioversity International's Regional Office for the Americas, in Cali, Colombia, in collaboration with three research units: l'Unité de Phytopathologie de l'Université catholique de Louvain, Louvain-la-Neuve, Belgium (UCL), the Laboratorio de Recursos Naturales, Unidad de Biología, Tecnología y Prototipos, Facultad de Estudios Superiores Iztacala de la Universidad Nacional Autónoma de México (UNAM), and the Laboratory of Gene Technology, Katholieke Universiteit Leuven, Belgium (KUL).

The main objective of the project was to study the genetic diversity within a plant host, the important forage legume genus *Stylosanthes*, in relation to the diversity found in its most important pathogen, the anthracnose fungus *Colletotrichum*, both collected from Mexico, the geographical area of origin of these organisms.

Located at the junction of two different biogeographical domains and showing a complex topography, Mexico is considered the second centre of diversity for *Stylosanthes,* and, as such, an ideal area for this study. *Stylosanthes* material collected in 17 Mexican states by UNAM, in combination with existing herbarium material, provided the basis for the macro- and micro-morphological, palynological, cytological, molecular and anthracnose studies.

Project components investigating the diversity of *Stylosanthes* and of its pathogen were implemented in Belgium by KUL and UCL, respectively. The research at UCL focused on the morphological, pathogenic and molecular characteristics of wild isolates of *Colletotrichum* associated with *Stylosanthes* in Mexico. These were compared with isolates causing anthracnose on cultivated *Stylosanthes* (mainly *C. gloeosporioides*). An accurate knowledge of wild isolates allowed clarification of their origin and their specificity for *Stylosanthes.*

KUL developed and used molecular markers for studies on inter- and intraspecific relationships in *Stylosanthes*. Since a solid taxonomic foundation was essential for such a study, UNAM conducted a systematic study of *Stylosanthes* in Mexico using macro- and micro-morphological, palynological and cytological data. Distribution patterns of all the Mexican species and their ecological requirements were also ascertained.

Bioversity International provided coordination and technical support, in particular providing guidance on germplasm collection and use. Based on the reports and scientific papers prepared by the partners, the strategy, methodology and the findings of each component are discussed.

2. Introduction

2.1 The host plant Stylosanthes

Stylosanthes species are among the most economically significant pasture and forage legumes in the tropical and subtropical regions, mainly because of their adaptation to a wide variety of climatic and edaphic conditions. They originate primarily from South and Central America. As a nitrogen-fixing legume, they restore soil fertility, and when used as cover crop they are known to stabilize the soil and arrest land degradation (Thomas 1984; Ramesh et al. 1997). *Stylosanthes* species can be used in both, grazed pastures and mixed crop-forage systems, and they have therefore a widespread application in a variety of animal feeding systems. In Asia and Africa, they are commonly used as freshly cut fodder, as dried hay, or as leaf meal supplement for fish and farm animals (Chakraborty 2004). In northern Australia, *Stylosanthes* serves as a protein-rich pasture plant for cattle. Some species appear to have the potential to substantially reduce populations of cattle tick (*Boophilus microplus*) because of the sticky secretions they produce, which immediately immobilize the larvae of the tick (Sutherst et al. 1982; Khudrathulla and Jagannath 1998).

Stylosanthes has achieved its greatest commercial success in countries outside its centre of diversity (Cameron and Chakraborty 2004; Pengelly et al. 2004). The first successes were obtained with the annual *S. humilis* and the perennial *S. guianensis* (Cameron and Chakraborty 2004). *S. scabra*, *S. hamata* (4x) and *S. viscosa* are other species that have been improved and of which commercial cultivars have been released. The majority of the other *Stylosanthe*s species are economically less important. They may, however, fill particular ecological niches or provide breeders with much needed genetic diversity for traits such as disease resistance and drought or cold tolerance.

The major constraint to *Stylosanthes* cultivation, wherever grown, is the anthracnose disease caused by the fungus *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc., a diverse and quickly adapting fungus. Anthracnose's threat to many productive *Stylosanthes* cultivars in many countries (Irwin and Cameron 1978; Irwin et al. 1984; Lenné 1993; Miles and Lascano 1997), has in the last decades led to intensified research for resistant lines. Genetic resources collection combined with evaluation and the resulting germplasm selections have continued to be the major contributors to new cultivars. Two important institutes working on *Stylosanthes* breeding are Australia's Commonwealth Scientific and Industrial Research Organisation (CSIRO) and the International Centre for Tropical Agriculture (CIAT) in Colombia. Both have included breeding programmes in addition to their germplasm activities (Schultze-Kraft 1984; Maass and Sawkins 2004).

There is no doubt that an improved taxonomic classification and an advanced understanding of the phylogenetic relationships between *Stylosanthes* species will enhance a better use of the germplasm.

2.1.1 Taxonomic history

The first time that a species now known to belong to the genus *Stylosanthes* was reported, was in 1696 when Sir Hans Sloane listed *Anonis non spinosa, minor, glabra, procumbens, flore lutea* as occurring in Jamaica (Sloane 1696). The plant's species name at that time was *Anonis non spinosa* and describing it as 'small, glabrous, procumbent, with yellow flowers', he was referring to the actual *Stylosanthes humilis* Kunth. In 1788, Swartz established the genus *Stylosanthes* Sw. with two species, *S. procumbens* (=*S. hamata* (L.) Taub) and *S. viscosa* (L.) Sw., and characterized it by the presence of a very long tubular calyx divided into five unequal and laciniate lobes, a papilionoid corolla, ten stamens with a free apex and a bi-articulate and uncinate pod as the fruit. Since that time, the genus has been revised several times. Vogel (1838) was the first to critically review the genus and to establish the main division of the

genus into the sections *Styposanthes* and *Stylosanthes*. Of the fifteen species mentioned by Vogel, seven were described for the first time (*S. angustifolia*, *S. leiocarpa*, *S. montevidensis*, *S. bojeri*, *S. scabra*, *S. capitata* and *S. bracteata*) and apart from *S. bojeri* which is now called *S. fruticosa*, the other six species are still recognized (Gama-López 2006). As new species were added by various authors, new infraspecific taxa emerged (Pilger 1901) and alternative taxonomic arrangements were proposed (Burkart 1939). Meanwhile, Blake described in 1920 seven new species and six new infraspecific taxa, of which three are endemic to Mexico, i.e. *S. macrocarpa*, *S. purpurata* (= *S. dissitiflora*) and *S. subsericea*. Mohlenbrock (1958) later changed the name of *S. purpurata* into *S. guianensis* ssp. *dissitiflora*. Before the actual division of the genus was established (Mohlenbrock 1960; 1963), the sections´ names (Herter, 1943) and their morphological traits were changed a couple of times (Mohlenbrock, 1958).

The most recent taxonomic treatments of the genus were undertaken by Ferreira and Costa (1979), who described 25 Brazilian species, and by 't Mannetje (1984) who published a new classification omitting the sections and using the length and fruit rostrum as main characters. During the latter study, 't Mannetje observed a lot of species difficult to identify taxonomically, e.g. the Mexican species *S. subsericea* and *S. macrocarpa*.

Since these last reviews, new Brazilian species have been described: *S. nunoi* (Brandão 1991), *S. longicarpa* (Brandão and Costa 1992), *S. seabrana* (Maass and 't Mannetje 2002) and *S. maracajuensis* (Costa and Van den Berg 2003). Although the taxonomic status of *S. seabrana* was only validated in 2002, commercial cultivars of this species had been registered already in 1996 (Maass and Sawkins 2004). *S. salina* (Costa and Van den Berg 2001) and *S. recta* (Vanni 1995) have recently been described for Mexico and Paraguay, respectively. *S. salina* was not considered in this research project, however, because results of morphological analysis showed that this species is a morphotype of *S. viscosa* (Gama-López 2006).

2.1.2 Taxonomic classification

Stylosanthes Sw. is a small neotropical herbaceous or suffruticose legume genus of the Leguminosae family (Lewis and Schrire 2003). Although for a long time it was classified in the *Aeschynomeneae* tribe, Klitgaard and Lavin (2005), now have firmly positioned the genus in the *Dalbergieae* tribe, based on analyses of three molecular DNA sequence data sets. The subfamily of *Stylosanthes*, Papilionoideae (or Faboideae) includes nearly 727 genera and 19 320 species, classified in 68 tribes (Polhill et al. 1981; Lewis et al. 2005). An estimated 50 genera and 850 species of papilionoids are found in tropical areas. About 2000 species of these can be used as forage crops, whereas others can be used as green manure as a nitrogen source (Williams et al. 1976).

Currently, the genus *Stylosanthes* contains an estimated 50 species. About 23 species are distributed in South America and 10 species are present both in South and Central America. Several Neotropical species have been cultivated in Africa, Asia and Australia (e.g. *S. guianensis* (Aubl.) Sw. s.l., *S. humilis* Kunth and *S. viscosa* Sw.) where they now are naturalized (Klitgaard and Lavin 2005).

The genus is characterized by sessile flowers, monadelphous stamens that are often united in a closed tube, dimorphic anthers, elongate receptacles, tri-foliate leaves, and stipules that are fused with the petiole. Fruits are loments that develop aboveground (Burkart 1939; Rudd 1981). The genus contains two subgeneric sections, section *Stylosanthes* and section *Styposanthes*. These are differentiated by the respective absence or presence of an axis rudiment, which is thought to be an aborted secondary floral axis (Taubert 1891), and the respective presence of one or two inner bracteoles (Mohlenbrock 1963; Kirkbride and Kirkbride 1987). Both sections include diploid species, but polyploid species are restricted to the section *Styposanthes* (Table 1).

The wide distribution of *Stylosanthes* and its interactions with different environmental conditions have resulted in the existence of polymorphic species, which has caused nomenclatural and taxonomic problems. This is reflected in the different views about the taxonomic treatment. The classical taxonomic treatment has been mostly based on fruit morphology, in particular, the length and degree of curvature of the pod beak, the indumentation of the pod, and the width and venation of the outer bract ('t Mannetje 1984). In contrast, Ferreira and Costa (1979) emphasized the number of vascular bundles, the leaflet venation and the growth habit as main diagnostic features when reviewing *Stylosanthes* from Brazil. As a consequence several members of the *S. guianensis* complex have been classified as distinct species by Ferreira and Costa (1979), while 't Mannetje (1977, 1984) classifies them as varieties (Table 2).

Table 1. Classification of *Stylosanthes* species into sections and according to their ploidy level.

Ploidy level[b]	Section *Styposanthes*	Section *Stylosanthes*
Diploid (2n=20)	*S. bracteata*	*S. acuminata*
	S. calcicola	*S. angustifolia*
	S. hamata	*S. aurea*
	S. macrocarpa	*S. biflora*
	S. macrocephala	*S. campestres*
	S. mexicana	*S. debilis*
	S. pilosa	*S. dissitiflora*
	S. seabrana	*S. gracilis*
		S. grandifolia
		S. guianensis
		S. hippocampoides
		S. hispida
		S. humilis
		S. leiocarpa
		S. microsoma
		S. montevidensis
		S. tomentosa
		S. viscosa
Tetraploid (2n=40)	*S. capitata*	
	S. fruticosa	
	S. hamata	
	S. mucronata	
	S. scabra	
	S. sericeiceps	
	S. subsericea	
	S. sundaica	
	S. sympodialis	
	S. tuberculata	
Hexaploid (2n=60)	*S. erecta*	

[a] According to 't Mannetje 1984; and Maass and 't Mannetje 2002.

[b] According to Atchison 1949; Cameron 1967; Stace and Cameron 1984, 1987; Vanni 1987; Vieira et al. 1993; Liu and Musial 1997; and Maass and Sawkins 2004.

Note that only species with a known ploidy level are included.

Table 2. Taxonomic treatment of the *Stylosanthes guianensis* species complex.

't Mannetje (1977, 1984)	**Ferreira and Costa (1979)**
S. guianensis (Aubl.) Sw.	
var. *robusta* 't Mannetje	*S. grandifolia* M.B. Ferreira & S. Costa
	S. aurea M.B. Ferreira & S. Costa
var. *intermedia* (Vogel) Hassl.	*S. hippocampoides* Mohlenbr.
	S. campestris M.B. Ferreira & S. Costa
var. *gracilis* (Kunth) Vogel	*S. gracilis* Kunth
var. *dissitiflora* (Robinson & Seaton) 't Mannetje	not considered
var. *longiseta* (Micheli) Hassl.	*S. longiseta* Micheli
var. *marginata* Hassl.	*S. acuminata* M.B. Ferreira & S. Costa
var. *guianensis*	*S. guianensis* (Aubl.) Sw. subsp. *guianensis*
	var. *vulgaris* M.B. Ferreira & S. Costa
	var. *canescens* M.B. Ferreira & S. Costa
	var. *pauciflora* M.B. Ferreira & S. Costa
	var. *microcephala* M.B. Ferreira & S. Costa

Additional methods to complement the use of morphological characteristics have been used to elucidate further the taxonomic relationships of *Stylosanthes*. 't Mannetje (1969) assessed affinities between *Rhizobium* and *Stylosanthes*; analysis of morphological and agronomical traits were conducted by Burt et al. (1971, 1974), karyotype and isozyme characterization by Stace and Cameron (1984) and biogeographical evaluation by Williams et al. (1984). Recent advances in molecular genetics and the development of molecular marker techniques have made important contributions to the phylogenetic, taxonomic and diversity research of *Stylosanthes*. The following techniques have been used by *Stylosanthes* researchers: randomly amplified polymorphic DNA (RAPD) (Kazan et al. 1993a,b; Glover et al. 1994; Vieira et al. 1997; Liu 1997; Gillies and Abbott 1998), restriction fragment length polymorphism (RFLP) (Curtis et al. 1995; Gillies and Abbott 1996; Liu and Musial 1995; 1996; Liu et al. 1999), amplified fragment length polymorphism (AFLP) (Sawkins 1999; Sawkins et al. 2001, Chang-Sun et al. 2004), DNA sequencing of the nrDNA internal transcribed spacer (ITS) region (Sawkins 1999) and sequence-tagged sites (STS) analysis (Liu et al. 1999; Liu and Musial 2001).

2.1.3 Phylogenetic relationships

To manage germplasm collections and to interpret the diversity of *Stylosanthes* for conservation practices, taxonomic and evolutionary studies are essential. Due to their agronomic importance, most studies have been concentrated on widespread, well known or agronomically important *Stylosanthes* species, while minor species have been neglected. However, many of these minor species occur in restricted or isolated areas with effective barriers to gene flow, which has allowed the evolution of regionally adapted genotypes (Stace and Cameron 1984).

The genus *Stylosanthes* has a monophyletic origin (Gillies and Abbott 1996) and is closely related to *Arachis* (Lavin et al. 2001). Gillies and Abbott (1996) were the first to undertake a detailed phylogenetic study of the genus. Based on RFLP analysis of chloroplast DNA in 18 *Stylosanthes* species, they proposed to divide *Stylosanthes* into 4 major clades. Liu et al. (1999) identified ten basal genomes in the *Stylosanthes* genus through the application of RFLP and STS analyses.

2.1.4 Allopolyploidy

Allopolyploidy may lead, as is the case for *Stylosanthes*, to the formation of new species. The presence of two divergent genomes in an allopolyploid species combines the traits of both progenitors, which usually contribute to generation of new genotypes adapted to a broader range of environmental conditions than their parents. Polyploids are generally hardier and more vigorous when compared to their parents (Lewis 1980). Some economically important cultivars are found in the tetraploid species. These are *S. scabra*, *S. hamata* [(*S. hemihamata* Stace & Cameron sp. nov. ined. (Stace and Cameron 1984)] and *S. capitata* (Maass and Sawkins 2004). Due to the occurrence of desirable traits such as disease and drought resistance, they have been cultivated worldwide as a forage plant (Edye 1997; Guodao et al. 1997; Lusembo et al. 1997; Miles and Lascano 1997; Ramesh et al. 1997).

The basic chromosome number of *Stylosanthes* is x=10. Most species are diploid (2x=20) but tetraploid (4x=40) species and one hexaploid (6x=60) species have been described. All known tetraploid species, with the exception of *S. capitata*, are thought to be the result of an intersectional hybridization between diploids, followed by polyploidization (Stace and Cameron 1984). As mentioned before, the *Stylosanthes* genus is divided into two sections, the section *Styposanthes* contains both diploid and polyploid species, while species in section *Stylosanthes* are exclusively diploid (Table 1).

RFLP and STS analysis of nuclear loci were used successfully to identify diploid progenitors for the allotetraploid species (Curtis et al. 1995; Liu and Musial 1997). The additive nature of RFLP and STS banding patterns enables the identification of the constituent genomes of allotetraploids. These molecular data revealed that all known allotetraploid species of *Stylosanthes* arose from hybridization between diploid species from section *Stylosanthes* (genomes B or C) and a diploid species from section *Styposanthes* (genome A) with the exception of *S. capitata,* whose putative ancestors, *S. macrocephala* or *S. bracteata* and *S. pilosa*, all belong to the *Styposanthes* section (Liu et al. 1999; Ma et al. 2004).

2.2 Colletotrichum gloeosporioides, *the major anthracnose fungus of* Stylosanthes

Colletotrichum gloeosporioides (Penz.) Penz. & Sacc. is considered the most important pathogen of *Stylosanthes*. This worldwide disease was first recorded at Deodoro in Brazil in 1937 on *S. humilis* (Anon. 1937). During the last decades this anthracnose fungus has considerably limited *Stylosanthes* cultivation in South America (Lenné and Calderon 1984; Miles and Lascano 1997), Florida (Sonoda et al. 1974; Lenné and Sonoda 1982a) and Australia. In the 1970s, anthracnose disease wiped out a quarter of the naturalized *S. humilis* growing on almost 2 million hectares in northern Australia (Ramesh and Gangaiah 2004). From the 1980s onwards, anthracnose caused extremely severe crop losses in Africa and isolates able to overcome resistance of newly released genotypes appeared rapidly (Maraite 1993).

The fact that the highly variable anthracnose pathogen eliminated some promising cultivars from the market gave rise to breeding strategies to develop cultivars with broad-based resistance. This, in turn, triggered extensive work on the epidemiology and genetics of the fungus. In general, commercial utilization of *Stylosanthes* has been more successful in countries outside the centre of origin (Miles and Lascano 1997), partly due to limited diversity in the pathogen population.

2.2.1 Morphology and sexuality

Colletotrichum gloeosporioides is characterized by an extreme variability of morphological and cultural characteristics (von Arx 1957; Mordue 1971; Sutton 1992). On potato dextrose agar (PDA), mycelium is mostly greyish white to deep grey. The underside of the colonies varies from white to dark green or brown, becoming darker with age. Conidia are usually

produced as pale salmon masses in acervuli, more often setose, but sometimes glabrous, and as much as 500 µm in diameter. Conidia are straight, hyaline, normally uninucleate, very variable in length (9-24 µm), usually 3–4.5 µm wide (Sutton 1980) up to 6 µm according to von Arx (1957), and present a variable proportion of obtuse apices. Appressoria are 6–20 ◈ 4–12 µm, ovoid to clavate, sometimes lobed. Conidia germinate and form appressoria at one or both extremities, at the end of very short or long hyphae. In culture media, appressoria are often formed from mycelium and become complex.

Many fungi reproduce both sexually and asexually. Often only one method of reproduction is observable at a specific point in time or under specific environmental conditions. In these cases, mycologists have devised two names: 'teleomorph' refers to the sexual phase of the particular fungus; 'anamorph' to the asexual phase. 'Holomorph' describes the 'whole fungus', encompassing both methods. In case of *C. gloeosporioides*, the teleomorph is *Glomerella cingulata* (Stoneman) Spaulding & Schrenk, for which very few data are available on optimal conditions required for its perithecial production. Perithecia, the fruiting bodies in ascomycetous fungi that contain the ascospores, are occasionally formed in young culture and more often in older ones. The perithecia are 85–300 µm in diameter, solitary or aggregated, globose, dark brown to black. The asci, (35)42–60(80) ◈ (8)10–12(14) µm, are clavate to cylindrical and thickened at the apex. They contain eight ascospores that are oval, or cylindrical to fusiform, unicellular and hyaline, (9)12–24(30) ◈ (3)4–6(8) µm (von Arx 1957). *G. cingulata* has first been described as homothallic by Edgerton (1914). Cisar et al. (1994) have demonstrated that self-sterile isolates of *C. gloeosporioides* from distantly related hosts were sexually compatible. Recent studies demonstrated the existence of a complex reproductive system in *G. cingulata*, involving a single locus but with multiple alternative alleles (Cisar and TeBeest 1999).

2.2.2 Taxonomy

The fungus is a heterogeneous and complex species, comprising several host-specific populations and exhibiting extreme morphological variability (Cox and Irwin 1988; Davis et al. 1992). Morphological characters such as shape and size of conidia, setae and appressoria have been used, together with host origin, to define *Colletotrichum* species. This has often induced confusion and led to a proliferation of unnecessary names, especially by excessive reliance on the host origin. von Arx (1957) grouped as many as 600 taxa under the species name *Colletotrichum gloeosporioides*. Sutton (1980) reduced the genus to 19 species and three "group species" (*C. gloeosporioides*, *C. dematium* and *C. capsici*) in which the variability was so important that an infraspecific level of classification was suspected, but at that time, there were not enough elements to support it. Sutton (1992) gathered partial descriptions for 17 additional species, but the lack of information on various morphological, cultural or pathogenic characteristics, as well as the possible synonymies, hampered a rigorous nomenclature classification.

In recent studies, the taxonomy of various *Colletotrichum* species, including intraspecific differentiation in *C. gloeosporioides* (Freeman et al. 1998, 2000; Abang et al. 2002) was re-examined in an attempt to integrate morphological, pathogenic and molecular data. The ITS1 and ITS2 regions have been used successfully in the genus *Colletotrichum* to elucidate phylogenetic relationships between species and to clarify their taxonomy (Sherriff et al. 1994; Sreenivasaprasad et al. 1996; Freeman et al. 2001; Hsiang and Goodwin 2001; Denoyes-Rothan et al. 2003; Martinez-Culebras et al. 2003).

2.2.3 Crop losses, epidemiology and management

The history of *Stylosanthes* cultivation gives evidence of the devastation that the anthracnose disease can cause worldwide. Anthracnose affects biomass and seed yield, and production losses in affected pasture lands can be up to 100% (Lenné 1986). A few assays have been

performed and demonstrated a reduction of 58% of the dry matter in *S. hamata* fields in Florida (Lenné and Sonoda 1982a) and of 64% to 100% in *S. guianensis* in Colombia (Lenné 1986). In Australia, the dry matter yield and the seed losses were, respectively, 22% and 16% in *S. scabra* cv. Fitzroy; 67% and 49% in *S. hamata* cv. Verano; and 53% and 42% in *S. guianensis* cv. Graham (Davis et al. 1987). In many Asian countries, anthracnose seriously affected the first commercial *Stylosanthes* cultivars (Guodao et al. 1997). Mohamed-Saleem and Adeoti (1989) stressed the need to identify more productive, anthracnose-tolerant *Stylosanthes* lines as alternatives to the cv. Verano stylo in West Africa. Between 1981 and the date of this latter publication, more than 300 *Stylosanthes* lines were introduced into Nigeria for screening.

Colletotrichum gloeosporioides, like all *Colletotrichum* species infecting legumes, is seed-borne (Ellis et al. 1976; Lenné and Sonoda 1982b; Davis 1987). Within pastures, raindrops and animal grazing are the main agents of anthracnose spread. In the epidemiology of this fungus, the sexual stage does not play a significant role. The asexually produced conidia are the principal inoculum source. The optimal conditions required for anthracnose development are temperatures between 20°C and 30°C and more than 12 hours of continuous leaf wetness (Irwin et al. 1984; Chakraborty et al. 1990). As the conidia in the acervuli are embedded in a mucilaginous matrix (Louis and Cook 1985), it requires free surface water to suspend the conidia and make them available for splash dispersal. Chakraborty and Datta (2003) observed an increase in fecundity (spores produced per lesion area) under a higher concentration of CO_2, and noted that this could lead to an increase in the spread and severity of disease in the future, having important implications for the functional duration of resistance in crop plants. A weather-based forecasting system for anthracnose severity on *S. scabra* was developed by Chakraborty et al. (2004). The model integrated 10 weather data attributes and was developed on the basis of artificial neural network (ANN) models. Briefly, the model allows one to predict anthracnose development at sites other than those where the data were collected, and allows calculation of the overall risk of anthracnose at field sites, using a set of weightings from the trained ANN models.

Due to the important seed losses and the susceptibility of juvenile plants, strategic application of fungicides such as benomyl could be applied for seed crops and at plant establishment (Irwin 1989). Davis (1987) showed that seedborne infections can be effectively prevented with benomyl when applied as a dry seed treatment. A study in India calculated the benefit of using fungicidal sprays to control anthracnose in *Stylosanthes*, showing that net returns were positive for all cultivars (Ramesh and Gangaiah 2004). Nevertheless, in extensive agricultural systems, such as in the huge cattle farms of Australia, or in developing countries, chemical control remains economically prohibitive.

The use of resistant varieties has thus been the principal recommended control strategy. The important genetic variability of *C. gloeosporioides,* however, has induced a rapid appearance of isolates able to overcome the resistance of the selected genotypes (Lourd et al. 1979; Maraite 1993). In Australia, the use of composites lines containing several resistance genes, such as cv. Siran (*S. scabra*), provides some insurance against new anthracnose isolates. Another strategy is intercrossing between species and subsequent selection of lines to develop a broader-based resistance. Inter- and intraspecific mixtures are also used in Australia. A more recent approach consists of pyramiding genes related to resistance through the use of linked DNA markers, but until now there have been no commercial applications of genetically engineered varieties (Partridge et al. 1996). Phylloplane bacteria may play a role in disease management in naturally existing conditions. Bacteria, particularly *Pseudomonas* spp. and *Bacillus* spp., collected from the phylloplane of *S. guianensis* in Peru, inhibited both mycelial growth *in vitro* and conidial germination of the anthracnose pathogen on leaves of *S. guianensis* (Lenné and Brown 1991).

2.2.4 Type A and type B anthracnose isolates

In the 1970s, pathogenic variation in *Colletotrichum gloeosporioides* was identified in Australia when two types were discovered, designated as type A and type B. They were isolated from *Stylosanthes* spp. and distinguished by the symptoms induced on plants, by infection strategy, by cultural and molecular characteristics and by host range (Irwin and Cameron 1978; Irwin et al. 1984). However, nowadays, the existence of strains that do not clearly belong to either type has been confirmed (Kelemu et al. 1999; Chakraborty et al. 2002).

Type A isolates infect a wide range of *Stylosanthes* species worldwide, causing restricted lesions characterized by a tan-coloured centre surrounded by a dark margin, 1–3 mm in diameter on leaves and 3–6 mm on stems (Irwin and Cameron 1978) (Figure 1a). Under warm and humid conditions, the lesions coalesce on leaves and petiole, causing defoliation, whereas lesions on stems develop into cankers (Sonoda et al. 1974) and can cause the death of susceptible plants. Mature acervuli are commonly observed in the centre of the lesions. Type B isolates are mainly restricted to *S. guianensis* and its symptoms are characterized by homogenous brown extensive lesions on leaves (Figure 1b) and on stems, causing defoliation, terminal shoot necrosis and plant death. These symptoms were also observed on *S. erecta, S. gracilis* and *S. grandifolia* (Lenné et al. 1983; Irwin et al. 1984).

Type A isolates usually produce conidia with obtuse apices, homogeneous in size, while type B isolates produce an important proportion of conidia with acute extremities, irregular in size. Additionally, type A grows more rapidly on solid medium than type B isolates (Irwin and Cameron 1978).

(a) (b)

Figure 1. Anthracnose symptoms of type A on *Stylosanthes fruticosa* (a) and of type B on *S. guianensis* (b). (Photographs courtesy of H. Maraite).

Differences in dsRNA patterns, karyotype and ITS regions and the use of neutral markers such as RAPD and AFLP have shown that the two types are genetically distinct, but that there is a considerable variation within each type. A clear distinction of the dsRNA patterns was demonstrated between type A and B isolates (Dale et al. 1988). Furthermore, transmission of dsRNA (via hyphal anastomosis) is unlikely to occur between the two types and the authors concluded that fusions between the types do not occur in the field. Important variation in chromosome size and number were observed, both between and within types A and B (Masel et al. 1990). The most important finding was that polymorphism was only observed for the mini-chromosomes. This suggests that, in the field, the genome of *C. gloeosporioide*s is very variable and that chromosomal re-arrangements should be considered as a means of generating variability for this fungus. Extensive studies on the diversity in South America has not only demonstrated a very important DNA polymorphism

between isolates from different localities, but also between isolates collected within the same area (Chakraborty et al. 1997; Kelemu et al. 1997, 1999; Weeds et al. 2003). RAPD allowed distinction of newly aggressive isolates of type A among a wide set of Australian isolates presenting a very low level of genomic diversity (Chakraborty et al. 1999).

2.3 Mexico, centre of diversity of the host and the pathogen

The centres of diversity and the natural distribution of *Stylosanthes* are well known. Its primary centre of origin is South America, while a secondary centre is located in the Mexico-Caribbean basin. Several species, however, are native to Africa and Asia (Williams et al. 1984).

The origin of the *Colletotrichum gloeosporioides* isolates that cause anthracnose on *Stylosanthes* remains unclear, although it is presumed that South America also corresponds to the centre of diversity of this pathogen (Kelemu et al. 2004).

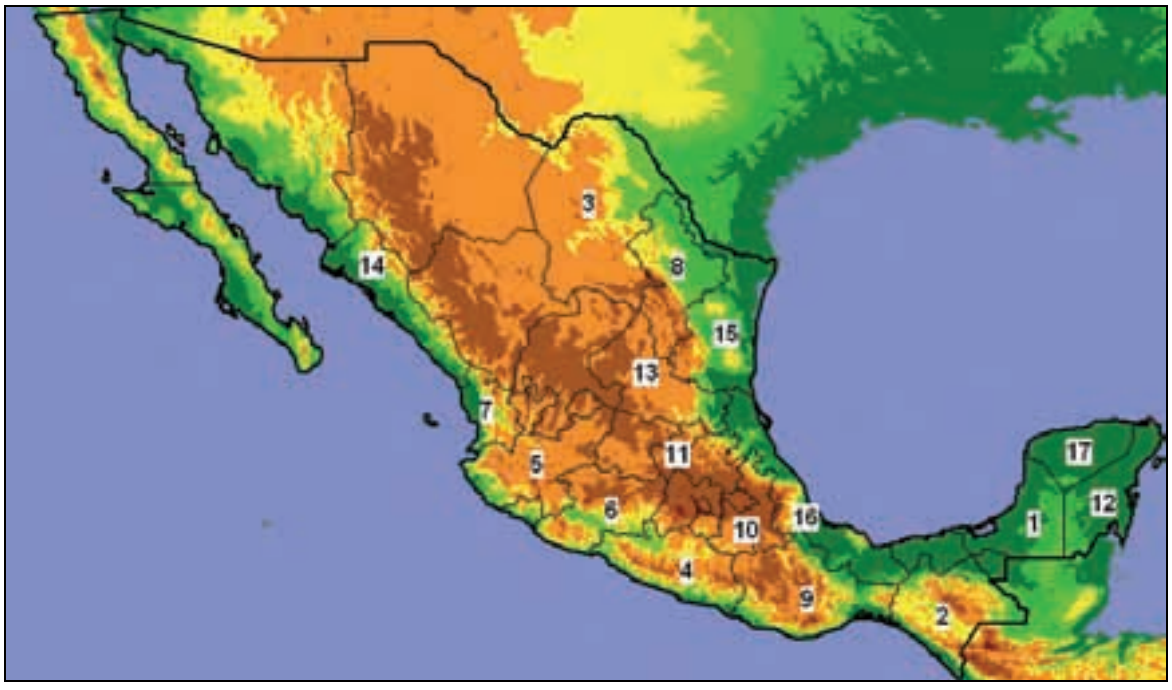
One hypothesis is that *C. gloeosporioides* isolates that are pre-existing in nature have passed to *Stylosanthes* crops and become progressively adapted to their new host. This could have been followed by the selection of aggressive isolates, which paved the way to the worldwide anthracnose epidemics. Another hypothesis is the occurrence of isolates specifically adapted to *Stylosanthes*, which have co-evolved with their host and which have been accidentally introduced into *Stylosanthes* growing areas. This suggests a correlation between the host and the pathogen diversity (Munaut 1999). According to Ramesh et al. (2004), the pathogen most probably has spread from its presumed centre of origin to the centres of *Stylosanthes* utilization through infected seed.

South American *C. gloeosporioides* isolates that infect *S. guianensis* demonstrated an important genetic and pathogenic diversity (Kelemu et al. 1997, 1999; Chakraborty et al. 2002; Weeds et al. 2003). Information on *C. gloeosporioides* present in wild *Stylosanthes* populations is limited, while occurrence of other *Colletotrichum* species in these populations has not been studied at all (Munaut 1999). In order to evaluate the diversity in the wild, where the fungus is not subjected to artificial selection pressure, the project reported here was carried out in Mexico. At the start of the studies, eight *Stylosanthes* species had been described in Mexico (Mohlenbrock 1958, 1963): *S. calcicola* Small, *S. guianensis* (Aubl.) Sw. [*S. guianensis* subsp. *guianensis* and *S. guianensis* subsp. *dissitiflora* (Robinson & Seaton) Mohlenbr.], *S. hamata* (L.) Taub, *S. humilis* Kunth, *S. macrocarpa* S.F. Blake, *S. mexicana* Taub., *S. subsericea* S.F. Blake, and *S. viscosa* Sw. As Mexico is located in the northern part of the *Stylosanthes* diversity centre, and as it shows a remarkable biological diversity and as it is characterized by a wide range of climatic, ecogeographical and edaphic conditions, this country seemed a natural candidate to host an significant representation of *Stylosanthes* diversity and corresponding *C. gloeosporioides* variability.

3. Systematic study of *Stylosanthes* in Mexico

The wide distribution of *Stylosanthes* and its ensuing interaction with different environmental conditions has resulted in the development of polymorphic species. This has resulted in a complex nomenclatural and taxonomic scenario for the genus. In an effort to shed light on this issue, a systematic study of the genus *Stylosanthes*, with a focus on the Mexican species, was carried out, integrating ecogeography, palynology, cytology, micro- and macro-morphology and molecular biology.

A thorough study of *Stylosanthes* in Mexico combined herbarium specimens with field collections. The herbarium species belonged to the collections of the Natural History Museum (BM), Instituto Politécnico Nacional, Herbario de la Escuela Nacional de Ciencias Biológicas (ENCB), Jardin Botanique National de Belgique (BR), Field Museum of Natural History (F), Harvard University (GH), Herbario del Instituto de Botánica, Universidad de Guadalajara (IBUG), Herbario Nacional de México, Universidad Nacional Autónoma de México (MEXU), Royal Botanic Gardens, Kew (K), Missouri Botanical Garden (MO), New York Botanical Garden (NY) and Smithsonian Institution (US). Collection took place during 15 field trips visiting 83 sites. Site selection was based on distributional data reported by Mohlenbrock (1958), 't Mannetje (1977), Williams et al. (1984) or derived from the labels of herbarium specimens. Sites were located in 17 states in Mexico, i.e. Campeche, Chiapas, Coahuila, Guerrero, Jalisco, Michoacán, Nayarit, Nuevo León, Oaxaca, Puebla, Querétaro, Quinta Roo, San Luís Potosí, Sinaloa, Tamaulipas, Veracruz and Yucatán (Map 1) (Figures 2 and 3). The material was collected, prepared, separated and packed for morphological, fruit micro-morphological, palynological, cytological, DNA and/or anthracnose study. All herbarium and field specimens were recorded in a database.



Map 1. States in Mexico where collection of wild *Stylosanthes* species took place. Key: 1. Campeche; 2. Chiapas; 3. Coahuila; 4. Guerrero; 5. Jalisco; 6. Michoacán; 7. Nayarit; 8. Nuevo León; 9. Oaxaca; 10. Puebla; 11. Querétaro; 12. Quinta Roo; 13. San Luís Potosí; 14. Sinaloa; 15. Tamaulipas; 16. Veracruz; and 17. Yucatán.

Figure 2. Specimen of *Stylosanthes macrocarpa* collected in the state of Oaxaca.

Figure 3. Specimens of *Stylosanthes guianensis* var. *occidentalis* Gama-López & P. Dávila var. nov. ined. collected in the state of Jalisco.

3.1 Description of Stylosanthes *species*

3.1.1 Morphology-based taxonomical study

During field collection, clear morphological polymorphism within some *Stylosanthes* species was observed. This polymorphism, together with the unclear taxonomical descriptions of the *Stylosanthes* genus and its corresponding species, complicated identification of the specimens collected. Therefore micro-morphology of *Stylosanthes* fruits and pollen was analysed, aimed at finding unique characteristics for each of the different *Stylosanthes* species. The results of this and the macro-morphological studies were used to undertake a phenetic analysis to discuss or to confirm the identity of the species and to analyse similarities between the species. Whereas at the start of this study eight species had been described for Mexico, when the project was finished eleven species were taken into consideration.

3.1.1.1 Macro-morphology

A first step in the identification of the collected specimens was to group them according to their morphological characteristics and by their geographical distribution to allow comparison with herbarium specimens. If the identity of the specimen could not be ascertained in this way, the use of the taxonomical descriptions by Mohlenbrock (1958) and 't Mannetje (1977) were not helpful either. These descriptions appeared to be unsatisfactory to determine the taxonomic classification of the often highly variable Mexican species.

The polymorphism within some species observed in the field was mostly related to the (eco)geographical distribution of the corresponding species. Variability in the widely distributed *Stylosanthes guianensis* was noticed such as in pubescence in the vegetative structures (viscid versus non-viscid), stem length, leaflet size and flowers per inflorescence. The wide geographical distribution of *S. viscosa* was also reflected in the different forms in its populations. 't Mannetje (1984) considered the length of the rostrum as an important trait to distinguish *S. viscosa* from other *Stylosanthes* species, describing it as having a rostrum less than 0.5 mm long. As Mexican field specimens, in contrast, often showed a rostrum longer than 0.5 mm, the length of the rostrum was not considered to be a consistent and differentiating character. Morphological differences in *S. dissitiflora* between the northern, central and southern Mexican populations were remarkable as well. Another example of polymorphism was detected in *S. humilis,* where populations growing on volcanic soils differed clearly from other populations of this species. Independently of the geographical region, or soil substrate, morphological variation within *S. macrocarpa* appeared to be very high, even within the populations. Another problem complicating identification was the very high similarity between some *Stylosanthes* species, such between as *S. mexicana* and *S. macrocarpa*.

Questions on species delimitations can often be resolved by phenetic analysis, a common tool in species identification. It also provides additional information on similarities between species as well as on the identification of the most discriminating morphological characters. To carry out the phenetic analysis, qualitative and quantitative characters of the vegetative and reproductive structures, such as stem, stipules, trichomes, leaflets, inflorescences, flowers, bracts, bracteoles and fruits, were measured from several plant specimens of each *Stylosanthes* species. The descriptor list applied consisted of 137 descriptors with 390 descriptor states (Gama-López 2006). A formal description of each of the 11 taxa present in Mexico has been elaborated as part of this taxonomic revision (Gama-López 2006).

3.1.1.2 Micro-morphology

Given the complex taxonomy of the genus, micro-morphology of *Stylosanthes* was also studied, i.e. fruit micro-morphology and pollen grains. Its main purpose was to determine species-specific morphological features of fruits and pollen grains that would facilitate solution of taxonomic problems in the genus.

Fruit micro-morphology

The number of papers documenting external micro-morphology of S*tylosanthes* fruits is limited. Serrato-Valenti et al. (1992) observed that the fruit pericarp is made up of prismatic crystals of calcium oxalate (idioblasts) and that it is covered by trichomes. No other detailed description was found of the external micro-morphology of *Stylosanthes* fruits.

In this study, the external micro-morphology of fruits from 20 herbarium samples, representing 11 Mexican Stylosanthes species, was examined in detail. Several parts of each fruit were observed, i.e. complete fruit, middle section of the fruit, apical region of the rostrum, fruit base and pubescence, to obtain a comprehensive micro-morphological description of each species (Gama-López 2006). Some external morphological features of the fruit of *S. macrocarpa* are illustrated in Figure 5. The density and size of the calcium oxalate crystals was found to differ for each species. All species showed uniserate trichomes with a unicellular or bicellular foot, with only *S. dissitiflora* possessing glandular trichomes.

Additional numeric data were obtained by measuring 5 external morphological features of 20 to 30 fruits per species, i.e. fruit length with rostrum not extended, fruit length with rostrum extended, fruit width, and length of both extended and not extended rostrum (Figure 4).

Results obtained by multiple analysis of variance revealed that the species *S. dissitiflora*, *S. guianensis* var. *guianensis* and *S. guianensis* var. *occidentalis* Gama-López & P. Dávila var. nov. ined. could be distinguished from the other *Stylosanthes* species by their small fruit width and their reduced rostrum. *S. viscosa* had a longer rostrum (either extended or not extended) than the previous three species, but the size of the fruit width was similar. The species *S. calcicola*, *S. humilis*, *S. macrocarpa*, *S. mexicana*, *S. pseudohumilis* Gama-López & P. Dávila sp. nov. ined., *S. quintana-roensis* Gama-López & P. Dávila sp. nov. ined., *S. subsericea* and *S. tehuacanensis* Gama-López & P. Dávila sp. nov. ined. were characterized by having a larger fruit width and a longer rostrum than all the other species examined (Gama-López 2006).

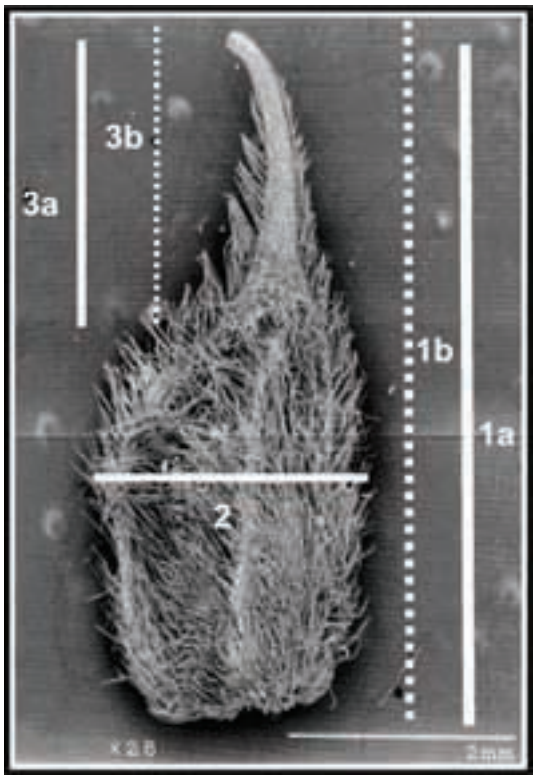


Figure 4. Measurements on each *Stylosanthes* fruit. 1a. fruit length with rostrum not extended; 1b. fruit length with rostrum extended; 2. fruit width; 3a. length of rostrum not extended; and 3b. length of rostrum extended.

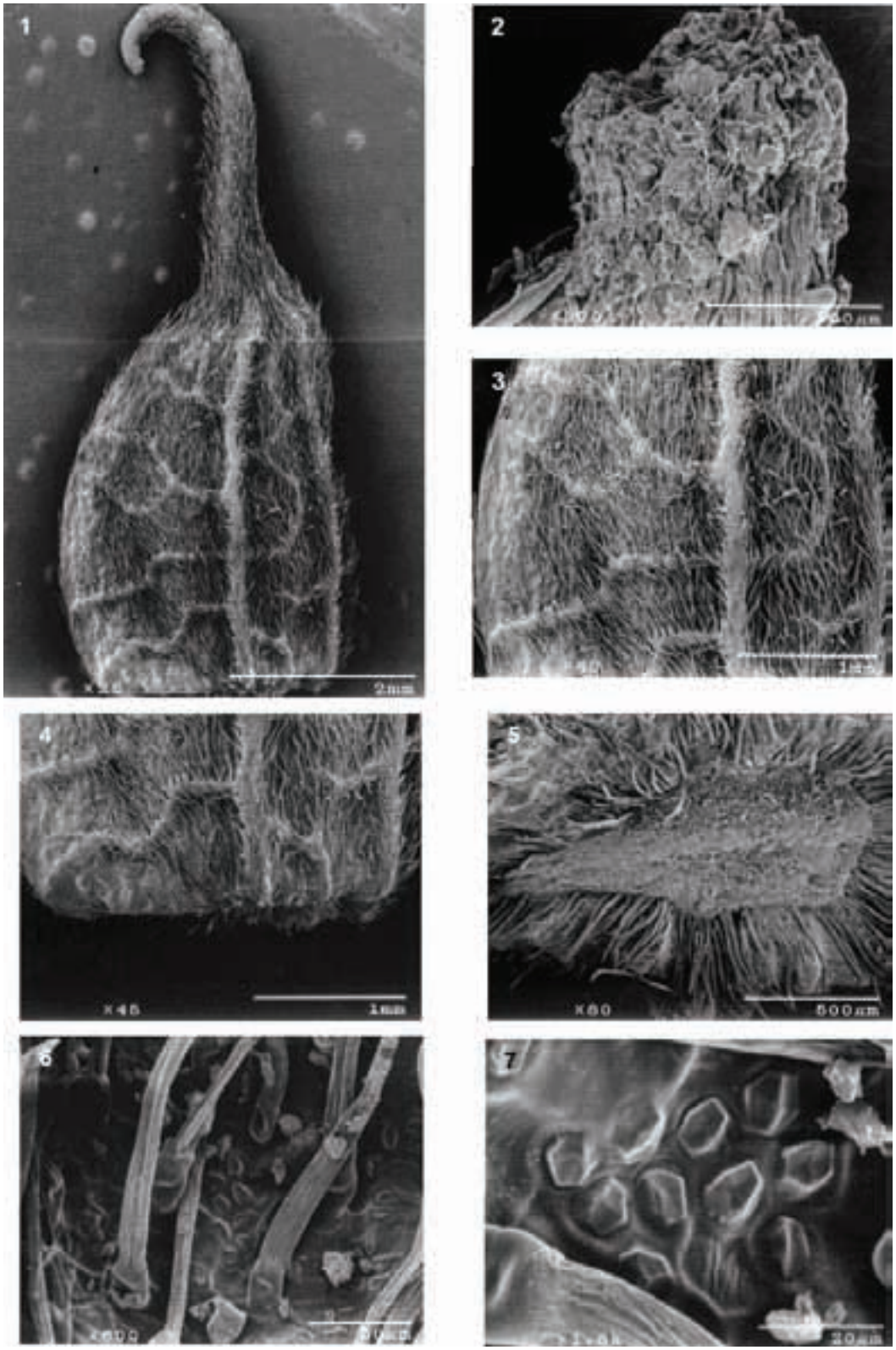


Figure 5. External morphological features of a *Stylosanthes macrocarpa* fruit. 1. complete fruit (25⋄); 2. apical region of the rostrum (500⋄); 3. middle section of the fruit (40⋄); 4. fruit base, lateral view (45⋄); 5. fruit base, bottom view (80⋄); 6. middle section, crystals of calcium oxalate and trichomes (800⋄); 7. extreme close-up of crystals (1800⋄).

Palynological study

Pollen grains of *Stylosanthes* were described for the first time by Melhem (1966), and later by Ohashi (1971) and Pire (1974). For most Mexican species, however, there were no analyses available. In addition to its contribution to the phenetic analysis, this study was also proposed as a contribution to knowledge of the pollen morphology of the Mexican *Stylosanthes* species.

Pollen samples were obtained from the anthers of herbarium samples and field-collected specimens belonging to 11 *Stylosanthes* species, including the two varieties of *S. guianensis* (*S. guianensis* var. *guianensis* and *S. guianensis* var. *occidentalis* Gama-López & P. Dávila var. nov. ined.). Fifteen pollen grains of each species were taken randomly. They were processed by the acetolysis method of Erdtman (1943), mounted with glycerine jelly to obtain permanent slides and observed through a light microscope (LM). For each species, the following characteristics of the pollen grains were measured: equatorial diameter (E), polar axis (P), and colpus width and exine thickness. These measures, as well as the derived measure of shape (P/E), were used to carry out an analysis of variance. Additionally, pollen samples were studied using a scanning electron microscope (SEM). Quantitative and qualitative characters obtained from these studies were analysed by multivariate analysis. Some characters are illustrated in Figure 6.

Analysis of variance of the values of the equatorial diameter, polar axis, colpus width, exine thickness and of the form coefficient showed that pollen grains of the different species are quite homogeneous, with exception of pollen size, where highly significant differences were observed at inter- and intraspecific level. S. pseudohumilis Gama-López & P. Dávila sp. nov. ined., S. quintana-roensis Gama-López & P. Dávila sp. nov. ined. and S. subsericea could be distinguished from the remaining species by the size of their pollen, which are bigger, i.e. a polar axis of 41.47–43.01 μm and an equatorial diameter greater than 26.98 μm. The exine thickness was also greater (more than 2.59 μm) and the coefficient P/E was between 1.33 and 2.00 (Gama-López 2006).

3.1.1.3 Phenetic analysis

All the data concerning the qualitative and quantitative characters of the macro-morphological study were included in the phenetic analysis. Two complementary methods were applied to carry out this analysis: hierarchical cluster analysis and principal component analysis (PCA), both by using the NTSYS-PC 1.8 software package. These methods evaluate the similarity of the *Stylosanthes* species as a function of a specific set of variables. The cluster analysis allows knowing the level of association between the different *Stylosanthes* specimens and species, based on similarities between the characters studied. PCA indicates identification of those characteristics that contributed most to the explanation of the differences between species and specimens.

Results of this phenetic analysis suggested the existence of 11 *Stylosanthes* taxa in Mexico, with 2 intraspecific taxa. At the beginning of the study 8 Mexican *Stylosanthes* species, described by Mohlenbrock were known (cf. Section 2.3 in this publication), i.e. *S. calcicola* Small, *S. guianensis* (Aubl.) Sw. [*S. guianensis* subsp. *guianensis* and *S. guianensis* subsp. *dissitiflora* (Robinson et Seaton) Mohlenbr.], *S. humilis* Kunth, *S. macrocarpa* S.F. Blake, *S. mexicana* Taub., *S. subsericea* S.F. Blake and *S. viscosa* Sw. Mohlenbrock (1958, 1963) recognized erroneously also the presence of *S. hamata* (L.) Taub. in Mexico (Gama-López 2006). Nevertheless, after having visited different *Stylosanthes* populations and identified the specimens collected, one could observe that the morphological characteristics of the supposed Mexican *S. hamata* populations had more morphological similarity to *S. humilis*. The taxonomic references consulted showed a lack of clarity in the taxonomic delimitation of the two species, as well as with respect to the phenology and the distribution intervals. Finally, at the end of this study, 11 *Stylosanthes* taxa and 2 intraspecific taxa were taken into

consideration. Besides *S. calcicola*, *S. guianensis* var. *guianensis*, *S. humilis*, *S. macrocarpa*, *S. mexicana*, *S. subsericea* and *S. viscosa*, a taxonomic combination was recognized for *S. dissitiflora* Robinson & Seaton. Moreover, three new species (suggested taxa) were detected, i.e. *Stylosanthes pseudohumilis* Gama-López & P. Dávila sp. nov. ined. (Figure 7), *Stylosanthes quintana-roensis* Gama-López & P. Dávila sp. nov. ined. and *Stylosanthes tehuacanensis* Gama-López & P. Dávila sp. nov. ined., and one new intraspecific taxon, namely *Stylosanthes guianensis* var. *occidentalis* Gama-López & P. Dávila var. nov. ined. (Gama-López 2006).

The results of the phenetic analysis also allowed a better understanding of the taxonomic delimitations of the Mexican *Stylosanthes* species. The most relevant character to distinguish the different Stylosanthes species was the shape of the fruit base, which allowed division of the 11 species into two sections: (1) Section Humilis, which corresponds to those species that possess a fruit base that is longer than wide; and (2) Section Viscosa, which includes the species that have a fruit base with equal length and width. Results of the phenetic analysis illustrated the importance of some morphological characteristics that were not considered relevant or important in previous taxonomical studies: exine thickness, colpus width, ornamentation of exine, fruit base, presence of glandular trichomes on epicarp, length of lateral calyx lobes, calyx width and length of outer bract. In general, the clusters obtained corresponded well with species delimitations. *S. calcicola, S. dissitiflora, S. humilis* and *S. viscosa* are well delimited species, as proven by well defined clusters. Delimitation of the species *S. macrocarpa* and *S. mexicana* based on phenetic analysis is difficult due to similar characteristics. However, their distributional range is different.

The new species *S. pseudohumilis* Gama-López & P. Dávila sp. nov. ined. was identified first in Oaxaca and Guerrero, where it grows sympatric with the species *S. humilis*. *S. pseudohumilis* Gama-López & Dávila sp. nov. ined. is morphologically similar to *S. humilis*, but differs from it by the presence of an axis rudiment and two internal bracteoles, and by its ploidy level, which is tetraploid (4x=40). The second newly described species, *S. quintana-roensis* Gama-López & P. Dávila sp. nov. ined., is similar to *S. calcicola* in having a fruit with a straight rostrum and the same number of inner bracteoles. It varies, however, from the latter by having persistent stipules on the whole stem length and by the absence of an axis rudiment. The last new species, *S. tehuacanensis* Gama-López & P. Dávila sp. nov. ined., is similar to *S. viscosa* in its habit, in the presence of spots on the leaflets and in the number of bracts and bracteoles, but it differs from *S. viscosa* in having an axis rudiment, a fruit with a circular base and a recurved rostrum.

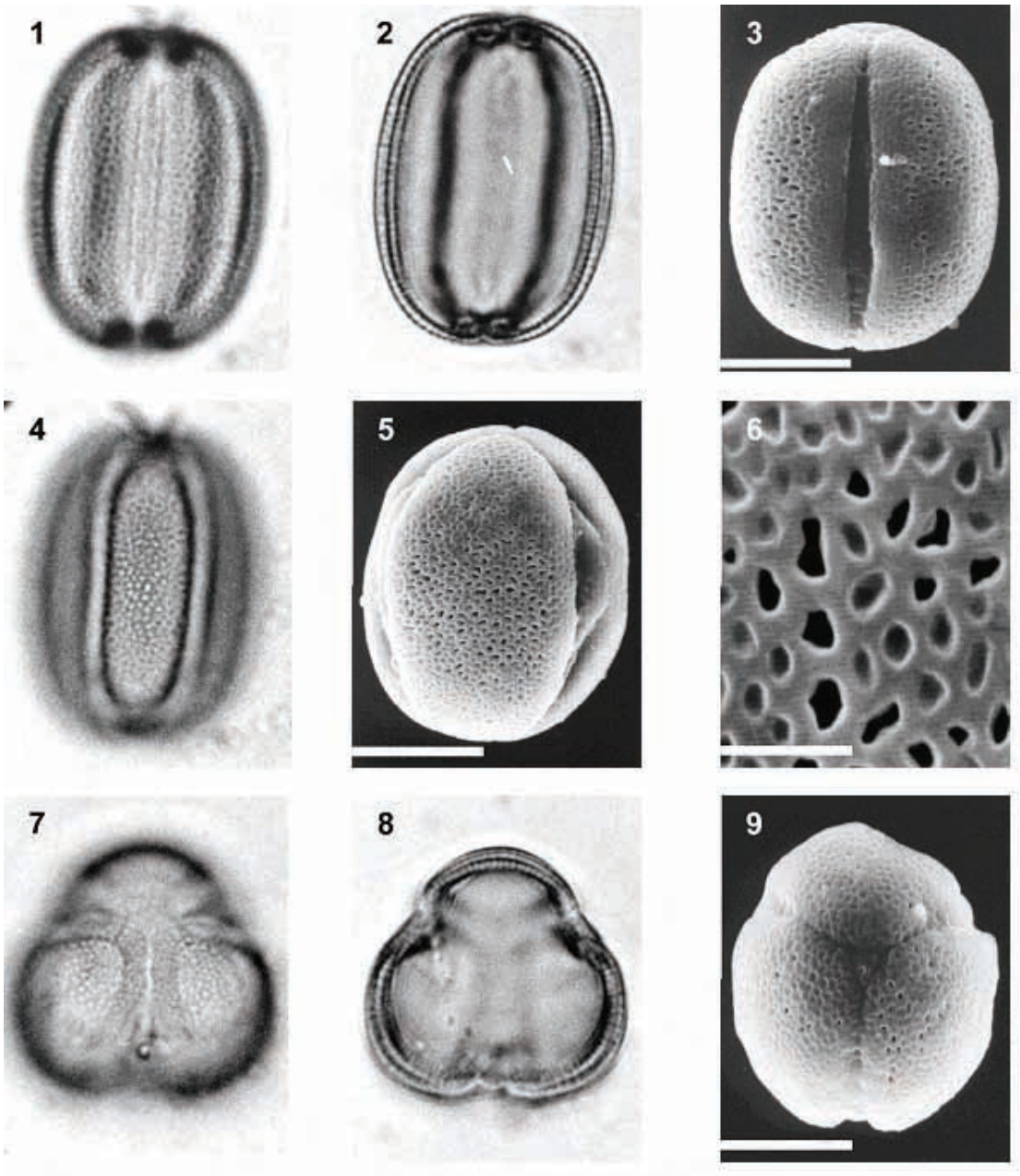


Figure 6. Ripe pollen of *Stylosanthes dissitiflora*. 1, 2 & 4 are pollen grains in equatorial view (LM) (1200⟐): 1. superoptic view; 2. optic section; and 4. infra optic view. 3 & 5 are pollen grains in equatorial view (SEM) (3000⟐, scale bar = 10 µm). 6 is a close-up of exine (SEM) (1500⟐, scale bar = 2 µm). 7 & 8 are pollen grains in polar view (LM) (1200⟐): 7. superoptic view; 8. optic section. 9 is a pollen grain in polar view (SEM) (3000⟐, scale bar = 10 µm).

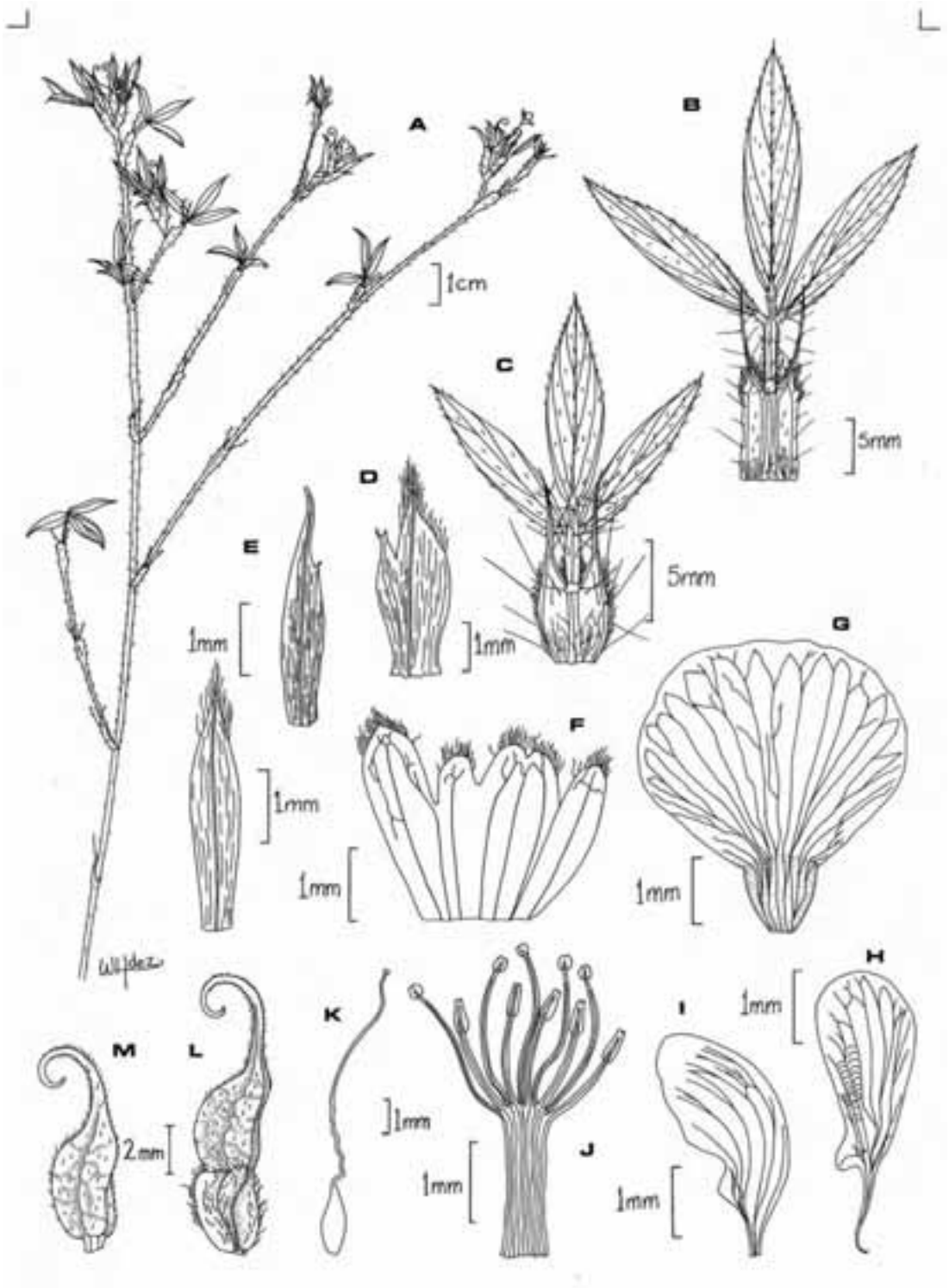


Figure 7. Plant parts of *Stylosanthes pseudohumilis* Gama-López & P. Dávila sp. nov. ined. Key: A. branch of an adult specimen; B. stipule; C. outer bract, trifoliate; D. external bracteole; E. internal bracteoles; F. calyx; G. standard; H. wing; I. keel; J. staminal tube with five dorsifixed anthers and five basifixed anthers; K. gynoecium; L. fruit a loment, bilocular, with both locules fertile; M. fruit with one fertile locule.

Based on the results of the phenetic analysis, UNAM elaborated a dichotomous key to identify the Mexican *Stylosanthes* species as a conclusion of their taxonomic revision. As the key had to be easily applicable, only directly observable and measurable characters were included.

1. Fruit base width and length are equal. 2
1. Fruit base wider than long, not equal. 5
   2. Rostrum uncinate. Inflorescence wider than long. Leaflets linear or lanceolate. 3
   2. Rostrum enrolled or incurved. Inflorescence longer than wide. Leaflets lanceolate, elliptic-lanceolate or elliptic-obovate. 4
      3. Fruit with glandular trichomes and with conspicuous venation. Leaflets linear to linear-lanceolate. Herbs erect or erect-prostrate. ***S. dissitiflora***
      3. Fruit without glandular trichomes and with inconspicuous venation. Leaflets lanceolate. Herbs prostrate or decumbent. ***S. guianensis***
      4. Rostrum enrolled. Axis rudiment absent. Leaflets lanceolate-elliptic or elliptic-obovate. ***S. viscosa***
      4. Rostrum recurved. Axis rudiment present. Leaflets lanceolate. ***S. tehuacanensis*** Gama-López & P. Dávila sp. nov. ined.
   5. Rostrum straight to recurved. Leaflets linear-lanceolate or lanceolate. 6
   5. Rostrum recurved, enrolled to straight-enrolled. Leaflets lanceolate, elliptic or obovate. 7
      6. Rostrum straight. Herbs erect, annual or perennial. Axis rudiment present. Stipules deciduous or if persistent only at the stem base. ***S. calcicola***
      6. Rostrum straight to recurved. Herb perennial to suffruticose sub-shrub or perennial sub-shrub. Axis rudiment absent. Stipules persistent on the whole stem. ***S. quintana-roensis*** Gama-López & P. Dávila sp. nov. ined.
      7. Recurved rostrum. 8
      7. Enrolled rostrum. 10
         8. Calyx tube minimum 10.0(-13.0) mm; standard minimum 8.0(-10.0) mm; keel minimum 8.0 mm. ***S. subsericea***
         8. Calyx tube maximum 10.0 mm. Standard and keel maximum 8.0 mm wide. 9
            9. Trifoliate bracts. Fruit glabrous or rarely pubescent. ***S. mexicana***
            9. Unifoliate and/or trifoliate bracts. Fruit pubescent. ***S. macrocarpa***
         10. Inner inflorescence bracteole 1(-2). Axis rudiment absent, occasionally present. Unifoliate bract and/or rarely trifoliate. Leaflets lanceolate to lanceolate-elliptic. ***S. humilis***
         10. Inner bracteoles 2. Axis rudiment generally present. Trifoliate bracts. Leaflets lanceolate to lanceolate-elliptic. ***S. pseudohumilis*** Gama-López & P. Dávila sp. nov. ined.

A key to identify the two *S. guianensis* varieties was also elaborated.

1. Stem with erect to prostrate growth, with non-viscid pubescence. Length of central leaflet maximum 45.0 mm, length of lateral leaflets maximum 40.0 mm. Length of inflorescence maximum 22.0 mm and maximum 25.0 mm wide, with more than 20 flowers per inflorescence. ***S. guianensis* var. *guianensis***
1. Stem with erect to prostrate growth, with viscid pubescence. Length of central leaflet maximum 36.0 mm, length of lateral leaflets maximum 30.0 mm. Length of inflorescence up to 15.0 mm and less than 15.0 mm wide, with less than 10 flowers per inflorescence. ***S. guianensis* var. *occidentalis*** Gama-López & P. Dávila var. nov. ined.

3.1.2 Distribution

For each Mexican *Stylosanthes* species, the general distribution is described according to Mohlenbrock (1958, 1960, 1963), 't Mannetje (1977) and Williams et al. (1984). In addition to the description of the distribution in Mexico and its corresponding habitat, distribution maps were prepared using the 642 geographical coordinates of the herbarium species and of the species collected. Maps were developed using ESRI® Arcmap version 9.0 (Gama-López 2006).

Genus *Stylosanthes* Sw.

General distribution: Widely distributed in tropical and subtropical regions.

Distribution in Mexico: In most of the Mexican territory: from the peninsula of Baja California to the state of Quintana Roo.

Habitat: In oak and pine-oak forest, in tropical deciduous, tropical evergreen and sub-evergreen forest, xerophytic scrubland, pasture (or savannah) and coastal dunes. Altitude varies from 0 to 2150 m.

Stylosanthes calcicola Small

General distribution: In the southeast of the USA and Mexico, as well as in Central America and the Antilles (Cuba, Dominican Republic and Jamaica).

Distribution in Mexico: In the Yucatán Peninsula and in the states of Campeche, Quintana Roo and Yucatán, as well as in Chiapas, where it was reported for the first time.

Habitat: Secondary vegetation of sub-evergreen and tropical deciduous forest, mangroves and coastal dunes. In disturbed areas next to road edges, in calcareous and alkaline soils and flood-prone areas. Altitude varies between 0 and 240 m, although in the state of Chiapas one population was found at 1550 m.

Stylosanthes dissitiflora Robinson & Seaton

General distribution: In Mexico and Central America.

Distribution in Mexico: This species had been described in the states of Jalisco and Chiapas. During the collection trips, new localities in the states of Sonora, Sinaloa, Nayarit, Guerrero and Oaxaca were reported.

Habitat: In pine-oak and oak forest and in disturbed areas of the oak forest. On ferric soils with sedimentary rocks. Altitude between 420 and 1350(-1680) m.

Stylosanthes guianensis (Aubl.) Sw.

General distribution: From Mexico down to northern Argentina.

Distribution in Mexico: From the state of Sinaloa to Chiapas.

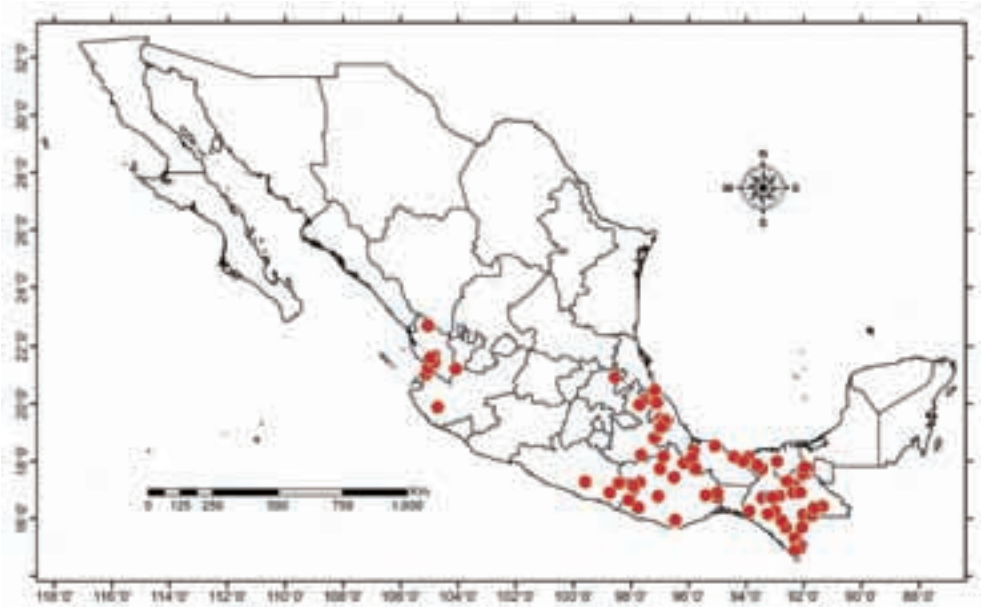
Habitat: At the borders of pine-oak forest, in evergreen and sub-deciduous tropical forest, in xerophytic scrubland and in pasture. It grows in ferric soils and occasionally on volcanic soils. Altitude between 250 and 1650(-1875) m.

Stylosanthes guianensis (Aubl.) Sw. **var. guianensis**

General distribution: Widely distributed on the American continent: from the northwest of Mexico to northern Argentina.

Distribution in Mexico: In the south, south-west and south-east of Mexico, i.e. in the states of Chiapas, Guerrero, Hidalgo, Jalisco, Nayarit, Oaxaca, Puebla, Tabasco and Veracruz (Map 2).

Habitat: At the edges of pine-oak forest, evergreen and sub-deciduous tropical forest, in xerophytic scrubland and pasture. It grows in ferric soils and occasionally on volcanic rock. Altitude between 250 and 1650 (-1875) m.



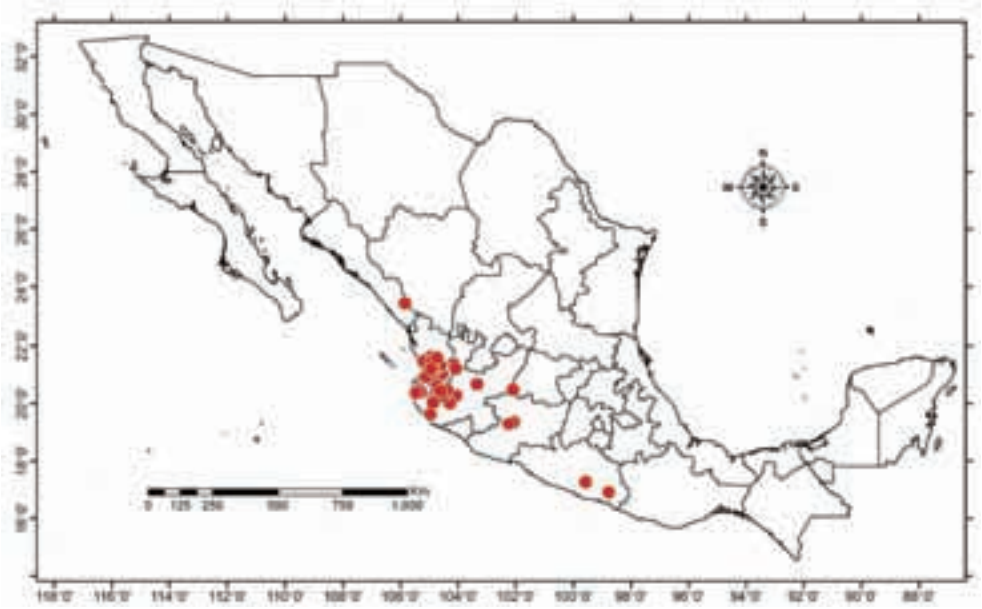
Map 2. Distribution of *Stylosanthes guianensis* var. *guianensis* in Mexico.

Stylosanthes guianensis* var. *occidentalis Gama-López & P. Dávila var. nov. ined.

General distribution: It is suggested that this is a new variety, probably endemic to Mexico (Gama-López, pers. comm.).

Distribution in Mexico: In the north-west and south-west of Mexico, in the states of Guerrero, Jalisco, Michoacán, Nayarit and Sinaloa (Map 3).

Habitat: In disturbed areas of oak and pine-oak forest. On ferric soils. Altitude between (470-)830 and 1690(-1860) m.



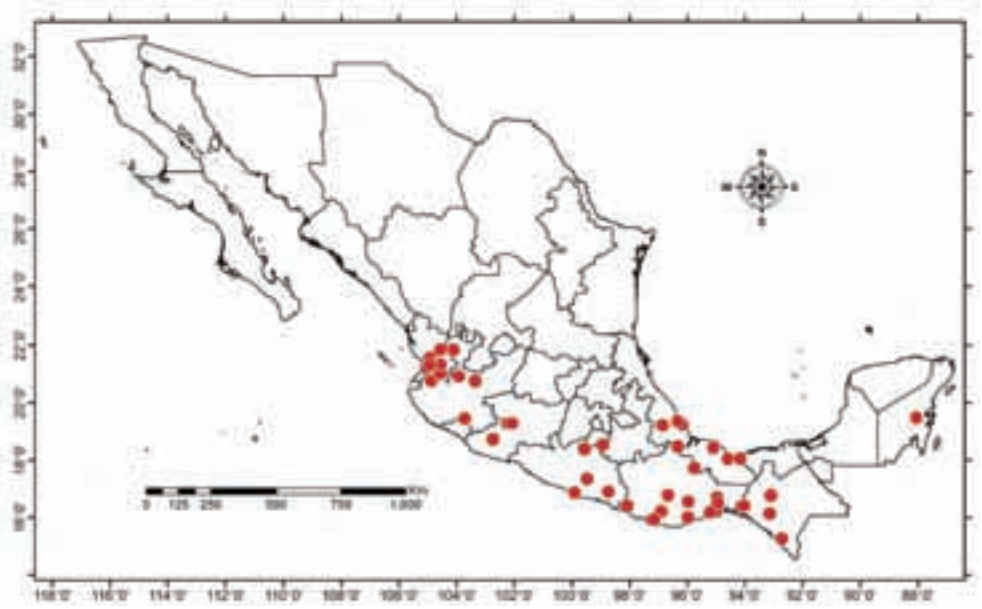
Map 3. Distribution of *Stylosanthes guianensis* var. *occidentalis* Gama-López & P. Dávila var. nov. ined. in Mexico.

Stylosanthes humilis Kunth

General distribution: In Mexico, Central America, the Antilles and South America.

Distribution in Mexico: In the south of Mexico, along the Pacific Coast and around the Gulf of Mexico, in the states of Chiapas, Colima, Guerrero, Jalisco, Michoacán, Morelos, Nayarit, Oaxaca, Quintana Roo and Veracruz (Map 4).

Habitat: Along the margins of oak and pine-oak forests and of tropical deciduous forest; in pasture, coastal dunes and on waste ground. In ferric and calcareous soils, and sometimes in volcanic soils, at altitudes up to 1600 m.



Map 4. Distribution of *Stylosanthes humilis* in Mexico.

Stylosanthes macrocarpa S.F. Blake

General distribution: Endemic in Mexico. Reported from Central America.

Distribution in Mexico: In the state of Oaxaca (south-east Mexico).

Habitat: It grows in the margins and disturbed areas of the pine-oak forest and in the margins of tropical deciduous forest. On ferric, rhyolitic and schist soils. Altitude ranges from 1500 to 1750(-1850) m.

Stylosanthes mexicana Taub.

General distribution: Found from Mexico down to Bolivia. The diploid genotypes are endemic to Mexico.

Distribution in Mexico: In the north-east of Mexico in the states of Coahuila de Zaragoza, Guanajuato, Hidalgo, Nuevo León, Querétaro de Arteaga, San Luis Potosí and Tamaulipas.

Habitat: At the edges of oak and pine-oak forest, and in xerophytic scrubland. On ferric and rhyolitic soils. Altitude from 300 to 1800(-2150) m.

Stylosanthes pseudohumilis Gama-López & P. Dávila sp. nov. ined.

General distribution: All *S. pseudohumilis* specimens are tetraploids. None of the herbarium specimens from Central and South America showed this character. Therefore this is suggested to be a new species, endemic to Mexico (Gama-López, pers. comm.).

Distribution in Mexico: From the north-west along the coasts of the Pacific Ocean and the Gulf of Mexico in the states of Chiapas, State of Mexico, Guerrero, Michoacán de Ocampo, Oaxaca, Puebla, San Luis Potosí, Sinaloa, Tamaulipas and Veracruz.

Habitat: *S. pseudohumilis* is part of the oak-pine and holm-oak forest and of the deciduous tropical forest. It is found in xerophytic scrubland and also in ruderal form on roadsides and in altered places, between 50 and 1340 m altitude.

Stylosanthes quintana-roensis Gama-López & P. Dávila sp. nov. ined.

General distribution: *S. quintana-roensis* is a newly described species.

Distribution in Mexico: *S. quintana-roensis* is only known in the south-east of Mexico, on the coast of Quintana Roo.

Habitat: *S. quintana-roensis* is part of the evergreen and sub-deciduous tropical forest. On sandy soils, at altitudes no higher than 10 m.

Stylosanthes subsericea S.F. Blake

General distribution: *S. subsericea* is endemic to Mexico.

Distribution in Mexico: In the south of Mexico, in the state of Oaxaca.

Habitat: At the edge of the tropical deciduous forest, at altitudes between 890 and 1050 m.

Stylosanthes tehuacanensis Gama-López & P. Dávila sp. nov. ined.

General distribution: *S. tehuacanensis* is a newly described species.

Distribution in Mexico: In the centre and the south of Mexico, in the states of Oaxaca and Puebla.

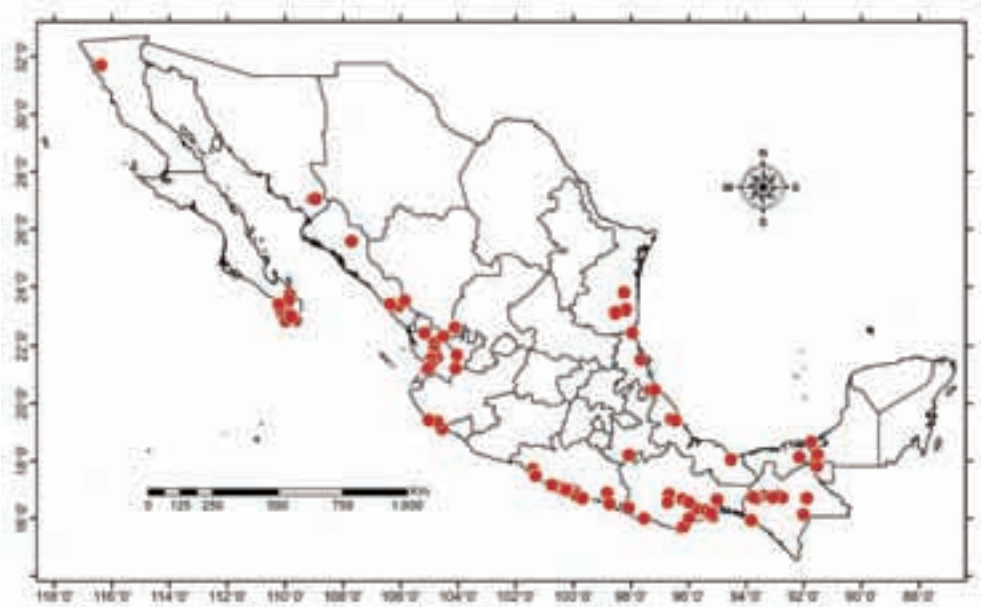
Habitat: In deciduous tropical forest and on roadsides. Between 800 and 2100 m altitude.

Stylosanthes viscosa Sw.

General distribution: Widely distributed on the American continent, from 29°S to 29°N latitude. In South America: mainly in Brazil, Paraguay, Guyana, Venezuela and Colombia. Widespread in all Central America up to northern Mexico, including the Antilles (Cuba, Dominican Republic and Jamaica).

Distribution in Mexico: In Baja California Sur (along the Pacific Ocean coast), in the states of Chiapas, Colima, Guerrero, Jalisco, Nayarit, Oaxaca, Sinaloa and Sonora, and on the coast of the Gulf of Mexico, in the states of Tabasco, Veracruz and Tamaulipas, the last-named being the most northern place where *S. viscosa* has been reported (Map 5).

Habitat: In oak forest and deciduous tropical forest, in xerophytic scrubland, coastal dunes and mangroves, also found in disturbed places and on roadsides in ferric to limestone soils, at altitudes up to 1710 m.



Map 5. Distribution of *Stylosanthes viscosa* in Mexico.

3.2 Cytogenetics

Stace and Cameron (1984) considered that in order to understand the polyploid populations of some *Stylosanthes* species, as well as to document their genetic origin, studies on the ploidy level should be prioritized.

Observation of the mitotic and meiotic chromosomes of the Mexican *Stylosanthes* species revealed the presence of diploid and tetraploid species with normal behaviour during meiosis. Differences in chromosome length, as well as in the number and position of secondary constrictions, were observed. The chromosome numbers of the Mexican *Stylosanthes* species are reported for the first time.

3.2.1 Mitosis

The chromosome numbers of the species were obtained from meristematic cells of primary roots. Roots were pre-treated with 0.001 M aqueous solution of 8-hydroxyquinoline for five hours. Subsequently, the chromosomes were stained using Feulgen´s solution (García 1990) with application of 1% aceto-orcein stain to enhance colouring. Permanent slides were obtained using the Conger & Fairchield method (Conger and Fairchield 1953).

The diploid and polyploid numbers of the species studied were 2x=20 and 4x=40, with a basic number of x=10 (Table 3). Results obtained are in agreement with Cameron (1967), Stace and Cameron (1984) and Vanni (1987). The very small chromosome size hampered the karyotype analysis, and the number of chromosome pairs with satellites in the terminal part of the arms could not be distinguished. Chromosome size data obtained from the mitotic cells of *S. dissitiflora*, *S. guianensis* var. *guianensis* and *S. guianensis* var. *occidentalis* Gama-López & P. Dávila var. nov. ined. varied, with the last two being larger.

Another important aspect was the clear difference between the polyploid cells of *S. pseudohumilis* and the diploid ones of *S. humilis*. In *S. pseudohumilis,* the chromosome number was always tetraploid (Figure 8), in contrast with *S. macrocarpa,* where diploid and tetraploid chromosomes were observed, although fewer tetraploids. This may correspond with the morphological variation of this species, and warrants further investigation.

Table 3. Chromosome numbers of Mexican *Stylosanthes* species.

Taxa	Population code	State	n	2n
S. calcicola	251	Yucatan		20
	252	Yucatan		20
S. dissitiflora	127	Jalisco	10	20
	281	Chiapas		20
S. guianensis var. *guianensis*	143	Veracruz	10	
S. guianensis var. *occidentalis*†	197	Nayarit	10	20
S. humilis	180	Oaxaca		20
	231	Nayarit		20
S. macrocarpa	178	Oaxaca		20/40
S. mexicana	246	Nuevo León		20
	248	Nuevo León		20
S. pseudohumilis†	136	Oaxaca		40
S. quintana-roensis†	254	Quintana Roo		40
S. viscosa	135	Oaxaca		20

NOTES: All specimens studied were collected by S. Gama-López and P. Dávila.

† S. Gama-López & P. Dávila var. nov. ined.

3.2.2 Meiosis

Haploid chromosome numbers were obtained from pollen mother cells. Chromosomes were stained with 1% acetocarmine and mounted with Hoyer´s solution (Anderson 1954) to obtain semi-permanent slides.

Studying the meiotic chromosomes of *Stylosanthes* species, it was confirmed that the haploid chromosomal number obtained (n=10), corresponded with half of the diploid number (2n=20). The meiotic behaviour was relatively normal. The chromosomal segregation occurred as expected with the separation of the ten chromosomes in each one of the poles in the first and second meiotic division. Meiotic cells presented at least one nucleolar organizer per genome and sometimes more than one bivalent was observed. There were only some irregularities observed in the mating of the chromosomes when becoming multivalent, probably due to chromosomic alterations (Stebbins 1971).

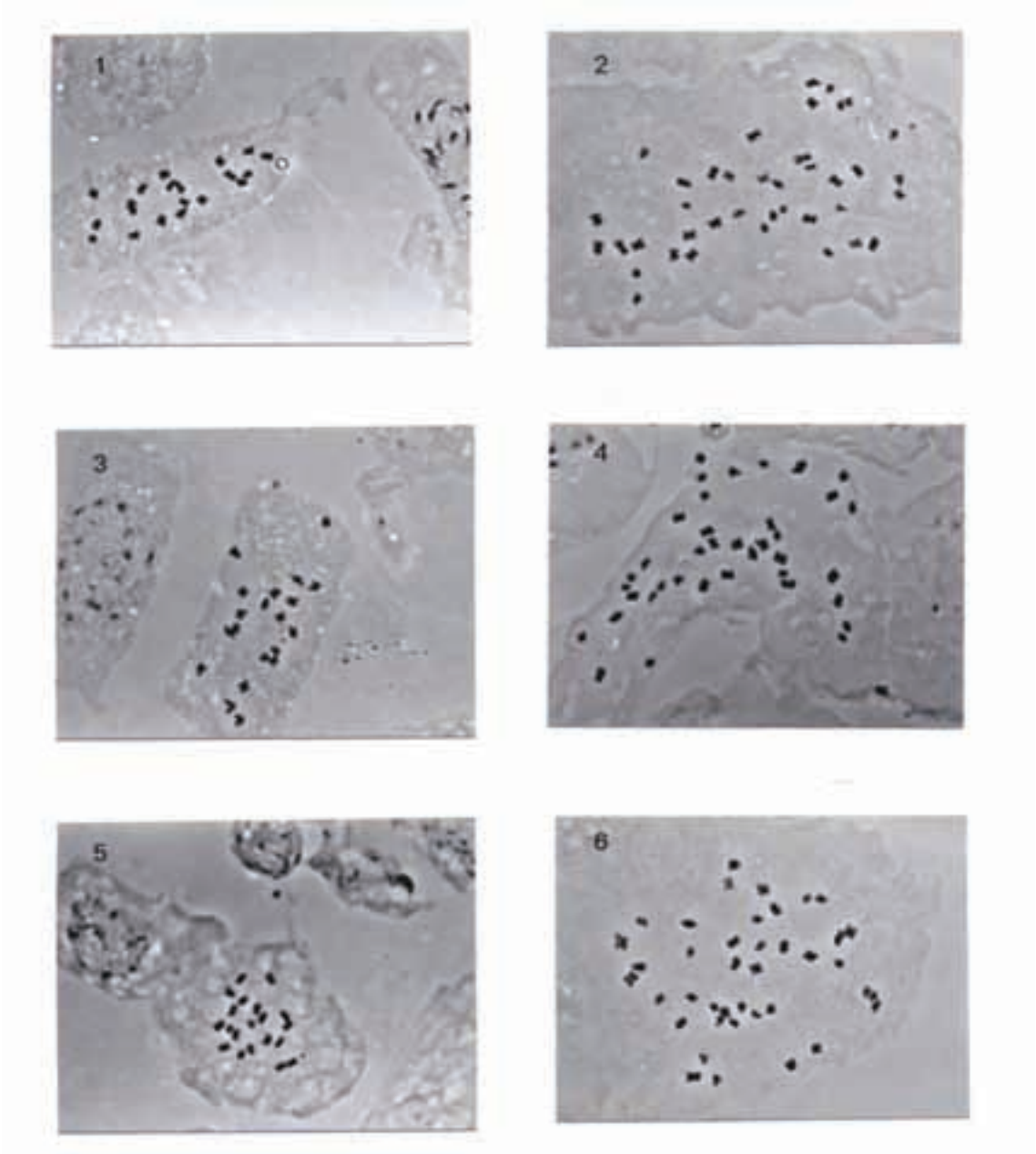


Figure 8. Chromosomes of two Mexican *Stylosanthes* species in mitosis. 1, 3 & 5: *Stylosanthes humilis*; 2, 4 & 6: *S. pseudohumilis* Gama-López & P. Dávila sp. nov. ined.

3.3 Phylogenetic analysis

Advanced understanding of phylogenetic relationships among *Stylosanthes* species, in combination with a well defined taxonomy, provides a solid basis for genetic diversity studies. In previous studies (Vander Stappen et al. 1999a,b, 2002a,b), phylogenetic relationships in *Stylosanthes* were inferred by DNA sequence analysis of the ITS region of nuclear ribosomal DNA and the *trn*L intron of chloroplast DNA (cpDNA) in 119 genotypes, representing 36 species of *Stylosanthes* and 7 species of the outgroup genera *Arachis* and *Chapmannia*. In this study, ITS and cpDNA sequences from 30 Mexican *Stylosanthes* genotypes representing 11 species and 12 taxa, were determined and compared to previously published sequences by multiple DNA sequence alignment, followed by parsimony analysis. The results are shown in Figures 9 and 10.

The Mexican genotypes of the diploid *S. dissitiflora*, *S. guianensis* var. *guianensis* and *S. guianensis* var. *occidentalis* Gama-López & P. Dávila var. nov. ined. are grouped in an exclusively diploid clade that contains all members of the *S. guianensis* complex, *S. montevidensis*, *S. macrosoma* and *S. biflora*. While this clade 4 as a whole is well supported, its inner structure is poorly resolved by the differences between *trn*L and ITS sequence types. The data does not answer the question of whether *S. dissitiflora* should be considered as a variety of *S. guianensis* ('t Mannetje 1977), as a subspecies (Mohlenbrock 1958) or as a different species (cf. Section 3.1.1.3 in this publication). Results from ITS DNA sequencing support the taxonomic study of Gama-López, namely that two distinct taxa of *S. guianensis* are present in Mexico: the well known variety *guianensis* and the newly recognized variety *occidentalis.*

The Mexican genotypes of the diploid *S. humilis* are clustered in clade 1, which contains diploid, tetraploid and hexaploid species. The occurrence of different ITS types in *S. humilis* may be attributed to the different morphological forms (Burt 1984) of this widely distributed species. The diploid species *S. viscosa* belongs to clade 1 for both sequences. Different ITS types were found in the widely distributed *S. viscosa*.

The tetraploid species *S. pseudohumilis* Gama-López & P. Dávila sp. nov. ined. clusters in clade 1 and 3 based on the *trn*L intron and the ITS region, respectively. The *trn*L sequence types of *S. pseudohumilis*, *S. humilis* and two genotypes of tetraploid *S. hamata* (C and E) are identical. *S. pseudohumilis* has a distinct ITS sequence type that is most similar to the ITS sequence types of diploid *S. hamata* genotype B from the Bahamas and tetraploid *S. hamata* genotypes C and E. Given the close morphological similarity between *S. pseudohumilis* and *S. humilis* (cf. Section 3.1.1.3 in this publication) and their shared chloroplast DNA haplotype, *S. humilis* is considered as the maternal progenitor of *S. pseudohumilis*. The paternal genome donor of *S. pseudohumilis* is related to the diploid species *S. hamata*. Remarkably, the allotetraploid genotypes of *S. hamata* from Venezuela and Colombia [*S. hemihamata* Stace & Cameron sp. nov. ined. (Maass and Sawkins 2004)] have their origin in a similar process of hybridization, with *S. hamata* s.str. and *S. humilis* as the putative paternal and maternal genome donors (reviewed in Maass and Sawkins 2004). However, both species have distinct morphological features, with *S. pseudohumilis* sp. nov. ined. resembling its maternal progenitor *S. humilis* (cf. Section 3.1.1 in this publication) and *S. hemihamata* resembling its paternal progenitor *S. hamata* s.str. (Stace and Cameron 1987). Most probably, independent hybridization events have taken place between geographically distinct genotypes of the widespread *S. humilis* and *S. hamata* s.str.

The diploid species *S. calcicola* clusters in clade 3, which is a clade containing diploid, tetraploid and hexaploid species of *Stylosanthes*. *S. mexicana* contains diploid as well as tetraploid genotypes (Vander Stappen et al. 2002a). The diploid genotypes, which are endemic to Mexico, are grouped in clade 3. The two genotypes of *S. mexicana* that were collected in Bolivia and Venezuela (Mohlenbrock 1958) are tetraploid (Vander Stappen et al. 2002a). The species *S. macrocarpa*, which is endemic to Mexico, is known to be diploid

(Vander Stappen et al. 2002a). However, in her taxonomic study, Gama-López identified diploid as well as tetraploid genotypes (cf. Section 3.2 in this publication). The diploid genotypes belong to clade 3.

Discordance between nuclear and cpDNA analyses of the tetraploid genotypes of *S. macrocarpa*, *S. mexicana*, *S. subsericea* and *S. tehuacanensis* Gama-López & P. Dávila sp. nov. ined. are explained by a process of allopolyploidization, with inheritance of the cpDNA of one parent, which is related to clade 3, and fixation of the ITS sequences of the other, i.e. *S. viscosa*. A similar process has been suggested for the Central American species *S. ingrata* and *S. hamata* (type of *S. eriocarpa* Blake, genotype D), the South American species *S. scabra*, *S. sericeiceps*, the Caribbean species *S. tuberculata,* and the African species *S. fruticosa* (Vander Stappen et al. 2002a). The morphological and molecular diversity in these species may be related to independent and recurrent formation of these species by hybridization between different forms of the widespread *S. viscosa* and a second progenitor that belongs to clade 3.

Based on parsimony analysis of the *trn*L intron and ITS DNA sequences, the Mexican tetraploid species *S. quintana-roensis* is clustered in clades 3 and 1, respectively. Molecular data from DNA sequencing of cpDNA and ITS combined with STS-PCR (Vander Stappen et al. 2002b) give strong support to S. *quintana-roensis* having an allotetraploid origin, with the maternal progenitor from clade 3 and the paternal contributor from clade 1.

In conclusion, this phylogenetic study shows that Mexico, as a secondary centre of diversity, has been an important geographical region for *Stylosanthes* speciation, especially to speciation by allopolyploidization. Of the 11 species that were collected in Mexico, 5 are (partially) tetraploid, i.e. *S. macrocarpa*, *S. pseudohumilis* Gama-López & P. Dávila sp. nov. ined., *S. quintana-roensis* Gama-López & P. Dávila sp. nov. ined., *S. subsericea* and *S. tehuacanensis* Gama-López & P. Dávila sp. nov. ined. The interaction of species from clade 3 (*S. hamata*, *S. mexicana*, S. *macrocarpa* and *S. calcicola*), which have a restricted distribution, with the widespread and polymorphic species *S. humilis* and *S. viscosa* from clade 1, has been very important for the origin of these Mexican allotetraploids. Five of these presumed genome donors are found in Mexico. Most of the taxonomic confusion in *Stylosanthes* can be related to independent and recurrent formation of these allotetraploid species by hybridization between different forms of the genome donors. A similar observation was made for the *S. scabra* complex (reviewed in Maass and Sawkins 2004).

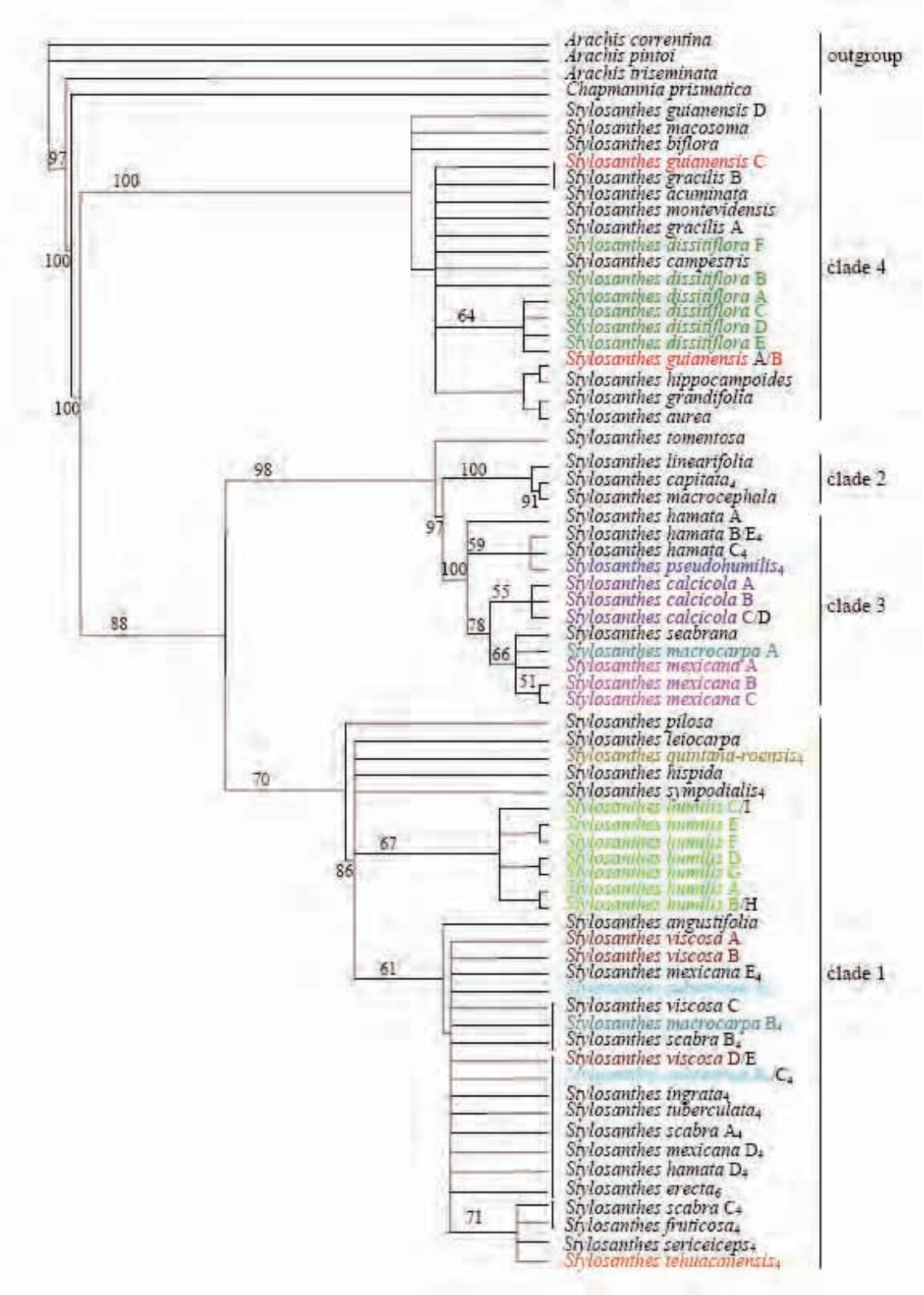


Figure 9. Consensus and semi-strict consensus of the most parsimonious trees obtained from parsimony analysis of the nrITS region. The Mexican genotypes are highlighted in colour. Horizontal bars before species names indicate identical sequences. For the species with more than one sequence type, the name of the species is followed by a letter code that refers to the genotype studied. Subscripts indicate ploidy levels.

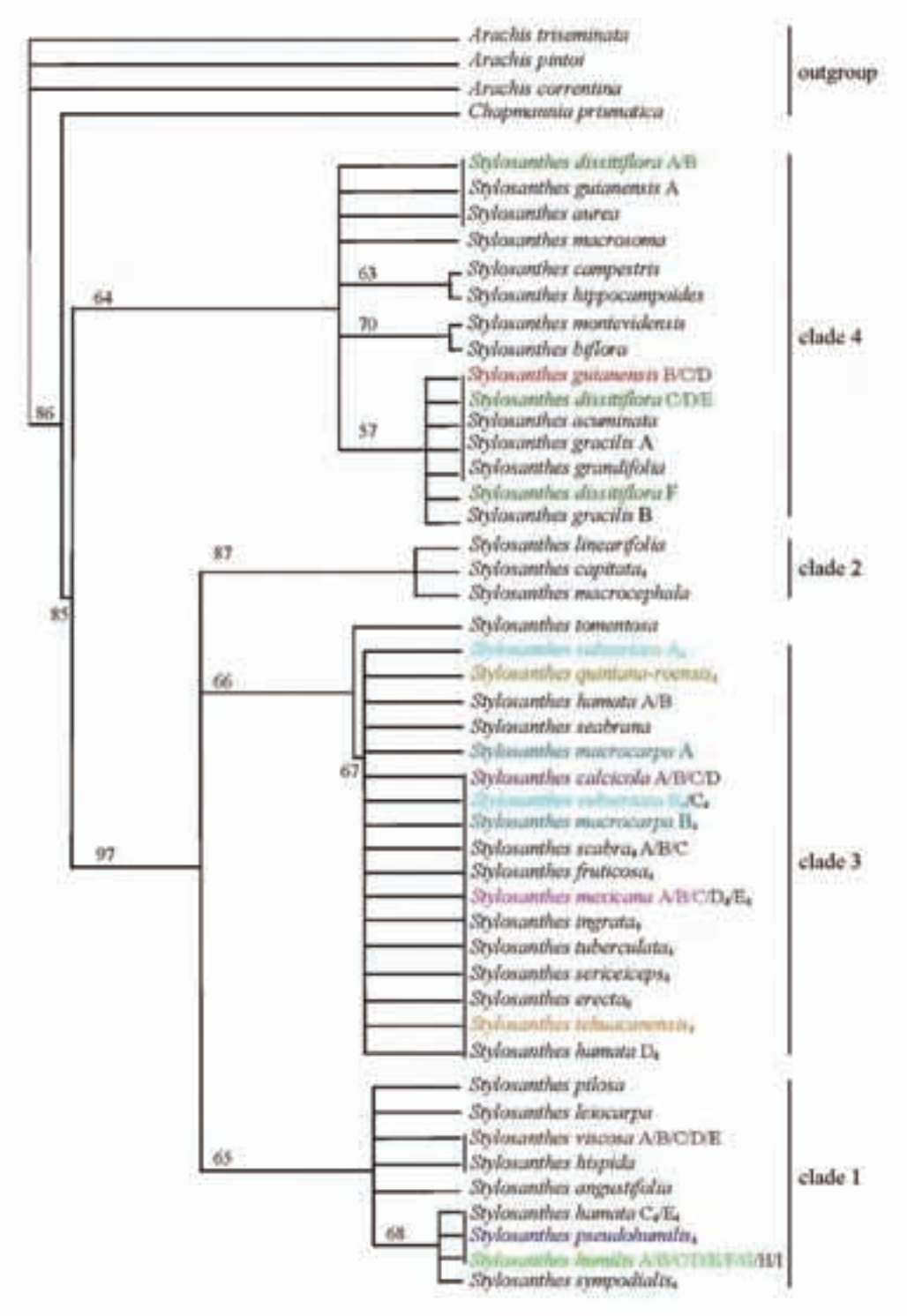


Figure 10. Consensus and semi-strict consensus of the most parsimonious trees obtained from parsimony analysis of the *trn*L intron region. The Mexican genotypes are highlighted in colour. Horizontal bars before species names indicate identical sequences. For the species with more than one sequence type, the name of the species is followed by a letter code that refers to the genotype studied. Subscripts indicate ploidy levels.

4. Genetic diversity of *Stylosanthes* species in Mexico

Knowledge of the genetic diversity and the genetic structure of populations is of great importance for conservation and germplasm management of the species. As Mexico, a centre of diversity for *Stylosanthes*, possibly has interesting genotypes for the search for anthracnose-resistant *Stylosanthes*, molecular tools are needed that can be used for a large-scale screening of the genetic diversity of natural *Stylosanthes* populations in Mexico. Besides a short presentation of the molecular tools used, the genetic diversity of four key *Stylosanthes* species in Mexico is discussed.

4.1 Molecular markers for screening genetic diversity in Stylosanthes *species*

Four PCR-based strategies were assessed to develop molecular markers in *Stylosanthes*. They differ from each other in several characteristics, such as (i) the target genome (mitochondrial, chloroplast or nuclear genome); (ii) the target DNA (coding or non-coding; single copy, low-copy or multi-copy; single-locus or multi-locus); (iii) the level of polymorphism; (iv) the nature of polymorphism (basepair differences or length variation); and (v) allele frequency information (dominant or co-dominant). Depending on these characteristics, each molecular technique is appropriate for one or more aspects of biodiversity studies.

In the first approach, DNA sequence analysis of two non-coding chloroplast regions, the *trn*L intron and the *trn*LF intergenic spacer, was used successfully in *Stylosanthes* to study species relationships and evolution (Vander Stappen and Volckaert 1999; Vander Stappen et al. 1999a–c, 2002b). In addition, chloroplast DNA sequence analysis was very useful for the identification of the maternal progenitors of polyploid species. The presence of a polymorphic microsatellite region and the observation of intraspecific variation in *S. humilis* makes the intergenic spacer region also suitable as a marker below the species level.

The second approach involved DNA sequencing of the nuclear internal transcribed spacer (ITS) region (Vander Stappen et al. 1998, 2002a,b). The DNA sequence data derived from ITS allowed us to determine evolutionary relationships in *Stylosanthes* (cf. Section 3.3 in this publication). Most polyploid species contained one ITS copy, with similarity to either the maternal or the paternal progenitor, enabling the identification of one of the parental genome donors. Intraspecific variation in the ITS DNA sequence was found in several species. This variation was consistent with the genetic variation inferred from other molecular data, demonstrating the usefulness of this marker for genetic diversity studies in *Stylosanthes*. Although DNA sequence information is highly valuable, the large-scale screening of populations by DNA sequencing is economically not feasible. As an alternative, the occurrence of basepair variations in specific DNA regions can be assessed by DNA sequence analysis of a limited number of samples, followed by searching for restriction sites and corresponding restriction enzymes using PCR-RFLP, which may reveal the determined (known) basepair variation in an indirect way by visualization on an agarose gel system.

The potential of the arbitrary-PCR techniques, i.e. RAPD and AFLP, in determining inter- and intraspecific variation in *Stylosanthes* species was tested and discussed (Vander Stappen 1999). These techniques have the advantage that no prior sequence information is required of the species under study. Because of problems with reproducibility and contamination, RAPD analysis was considered to be less suitable for diversity studies. In contrast, AFLP analysis of *S. humilis* revealed that this technique is very useful for the determination of genetic variation at the individual level (Vander Stappen et al. 2000). At the species level, AFLP can be used in *Stylosanthes* to classify genotypes by species, to identify polyploid species in relation to their progenitors and to determine relationships between closely related *Stylosanthes* species (Vander Stappen 1999). Although AFLP is a very powerful molecular tool for genotyping, the technique cannot be accurately used to study the genetic structure of populations, because the different alleles cannot be distinguished from each other due to the

dominant character of the polymorphic AFLP bands.

The last approach involved the development and use of co-dominant PCR markers by site-targeted PCR analysis. This approach requires the design of primers that specifically target highly variable regions in the genome. One strategy for obtaining co-dominant markers was the isolation, characterization and conversion of microsatellites into single sequence repeat (SSR) markers (Vander Stappen et al. 1999c). Another strategy involved the design of sequence-tagged site (STS) primer pairs, which were derived from coding and non-coding regions of published *Stylosanthes* genes (Vander Stappen et al. 1999b). The site-targeted PCR markers were useful to determine intra- and interspecific variation, and to identify the origin and parentage of allotetraploid species by length and/or DNA sequence variation (Vander Stappen et al. 1999b, 2002b). A combined use of SSR and STS markers provides useful indirect measures for population parameters.

A summary of the relevance of the different DNA markers for studying genetic variation within and among *Stylosanthes* species is provided in Table 4. The information presented in the table is based on our own experience and is expected to be of great usefulness to investigators planning future genetic diversity studies in *Stylosanthes*.

4.2 Genetic diversity in the Stylosanthes guianensis *complex*

In her taxonomic study of *Stylosanthes* of Mexico, Gama-López distinguished three different taxa in the *S. guianensis* complex, i.e. *S. dissitiflora* Robins. & Seat, *S. guianensis* var. *guianensis* (Aubl.) Sw. and *S. guianensis* var. *occidentalis* Gama-López & P. Dávila var. nov. ined. (cf. Chapter 3 in this publication). The two varieties of *Stylosanthes guianensis* in Mexico have a distinct geographical distribution, i.e. var. *occidentalis* is mainly distributed in the north-west of Mexico and var. *guianensis* is mainly distributed in the south, south-west and south-east of Mexico. Nayarit is the only state where both types have been observed at the same location in the field. The species complex of *S. guianensis* is the most widely distributed species of the genus *Stylosanthes*. Its natural distribution extends from Mexico to Argentina ('t Mannetje 1977), whereas *S. dissitiflora* grows at the extreme northern geographical limit of the species complex (Williams et al. 1984).

4.2.1 Molecular characterization

Plant and seed material of 77 specimens belonging to the *Stylosanthes guianensis* species complex were collected by Gama-López in 8 Mexican states, i.e. Chiapas (2), Guerrero (1), Jalisco (20), Michoacán (5), Nayarit (36), Oaxaca (6), Sinaloa (1) and Veracruz (6). Of these, 71 belong to the species *S. guianensis* and 6 to *S. dissitiflora*. The *S. guianensis* collection contains the varieties *guianensis* and *occidentalis*.

Table 4. Usefulness of different molecular markers for genetic diversity studies in *Stylosanthes*.

	Consensus PCR		Arbitrary PCR		Site-targeted PCR	
	cpDNA	**ITS**	**RAPD**	**AFLP**	**SSR**	**STS**
Between species						
Taxonomy	++	++	+	+	++	++
Evolutionary relationships	++	++	–	-	+/–	+/–
Polyploidy - Identification	–	+/–	–	+	++	++
- Origin and parentage	++	++	–	+	++	++
Within species						
Genotyping	–	+	+/–	++	++	++
Population genetics	+	+	+/–	+	++	++

Key to symbols: ++= very good; += good; +/- = appropriate but not recommended; and – = not appropriate.

4.2.1.1 Chloroplast and nuclear DNA sequence analysis

The DNA sequencing of the chloroplast *trn*L intron and the ITS region in the genotypes studied revealed distinct cpDNA haplotypes or ITS sequence types, or both, enabling discrimination between the three taxa identified in the morphological study by Gama-López: *Stylosanthes dissitiflora* and the two distinct subgroups of *S. guianensis*, i.e. the varieties *guianensis* and *occidentalis*, respectively (cf. Section 3.3 in this publication). The Mexican varieties of *S. guianensis* can be clearly separated from each other by PCR-RFLP (Figure 11).

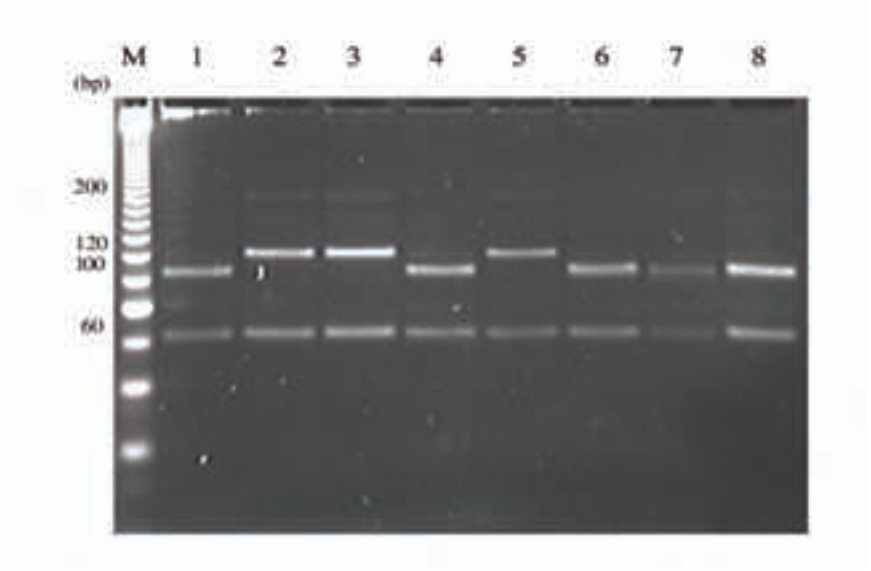


Figure 11. Restriction site analysis of PCR products amplified by primer pair ITS5-ITS2 and digested by *Bsr*FI in the eight genotypes of *Stylosanthes guianensis* studied. Lane M is the DNA size marker.

4.2.1.2 Site-targeted PCR markers

The Mexican collection of *Stylosanthes guianensis* and *S. dissitiflora* was analysed using 18 SSR and 9 STS primer pairs, previously found to be variable within the *S. guianensis* complex (Vander Stappen 1999; Vander Stappen et al. 1999c). The primer pairs were useful in discerning *S. dissitiflora* from *S. guianensis*, and *S. guianensis* var. *guianensis* from var. *occidentalis*, and revealed polymorphism within each taxon. Hybrids were found between *S. dissitiflora* and *S. guianensis* var. *guianensis*, and between *S. guianensis* var. *guianensis* and var. *occidentalis*, suggesting the occurrence of interspecific hybrids in the *S. guianensis* complex. These hybrids show additivity of PCR fragments for each marker used, representing the combination of two different genomes (Figure 12).

Within *S. guianensis* var. *guianensis* and var. *occidentalis*, additivity of alleles was observed for several site-targeted markers. The occurrence of heterozygotes indicates that some degree of outcrossing is present within each of the *S. guianensis* varieties. Similarity values between the genotypes were calculated based on absence/presence of length variants from 25 site-targeted PCR markers and subsequently used in principal coordinate analysis to depict the relationships within the Mexican *S. guianensis* complex by a 2-dimensional plot (Figure 13). The figure shows that the *S. guianensis* complex in Mexico is resolved into three major groups that correspond to the taxonomic identification of Gama-López (cf. Section 3.1.1.3 in this publication). The interspecific hybrid genotypes (G119-2, G257-1 and G265-1) are intermediate to the groups that contain the parental genome donors of these genotypes.

4.2.2 *Stylosanthes guianensis*

4.2.2.1 *Stylosanthes guianensis* var. *guianensis*

A total of 45 genotypes representing 27 specimens of the Mexican *Stylosanthes guianensis* var. *guianensis* collection of Gama-López were analysed by site-targeted PCR analysis to assess intraspecific variation, the distribution of genetic variation and the occurrence of heterozygotes. The genotypes were collected in the states of Chiapas (3), Guerrero (2), Nayarit (18), Oaxaca (7) and Veracruz (15).

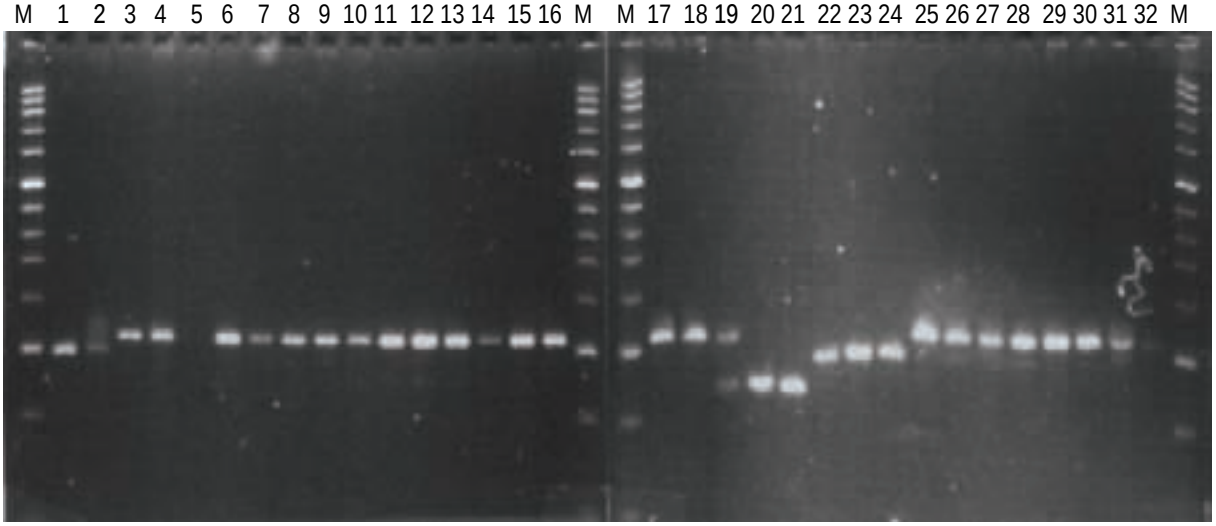


Figure 12. PCR products amplified by primer pair SSR1-24F/R in the 32 genotypes of the *Stylosanthes guianensis* complex. Lane M is the DNA size marker. Lanes 2 and 19 correspond to the hybrids.

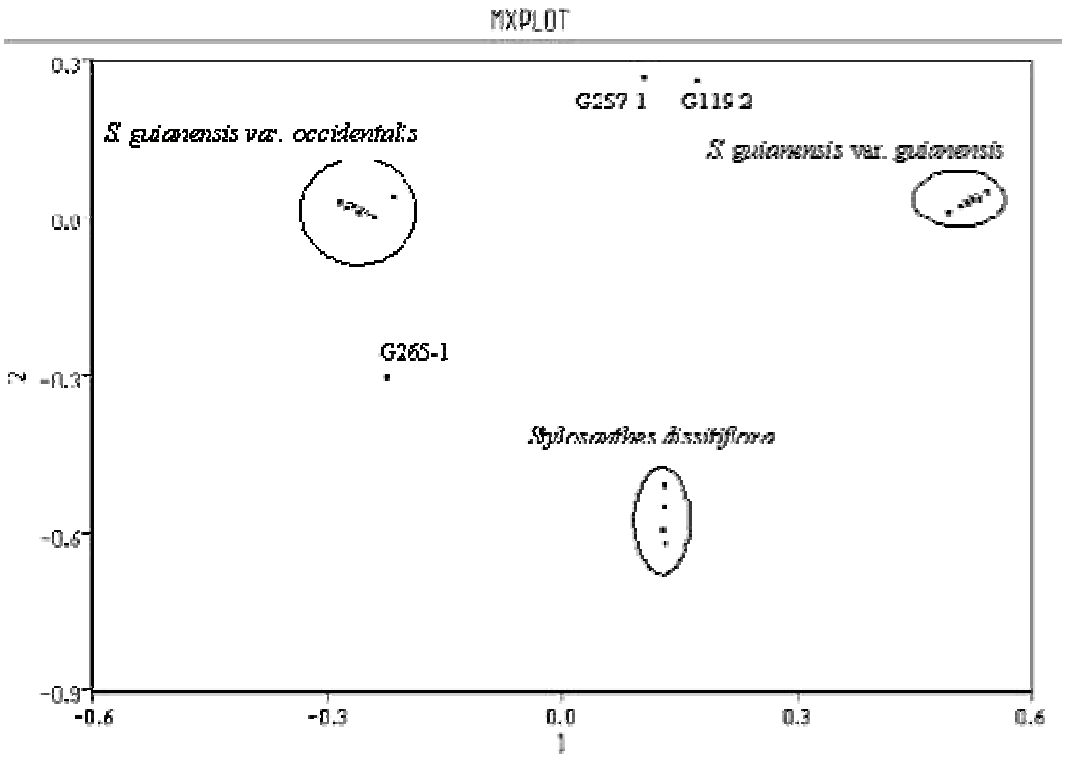


Figure 13. Principal coordinates analysis (PCA) of similarity data from site-targeted PCR analysis of *Stylosanthes guianensis* and *Stylosanthes dissitiflora*.

Five site-targeted PCR markers were found to be polymorphic in length among the *S. guianensis* var. *guianensis* genotypes. All these markers amplified one locus, enabling the identification of heterozygotes. The proportion of heterozygotes ranged from 2 to 15%. Based on the polymorphisms, genetic distances among the Mexican genotypes of *S. guianensis* var. *guianensis* were calculated and visualized by a two-dimensional plot (Figure 14). The figure shows that there is no strict subdivision within the Mexican *S. guianensis* var. *guianensis* according to geographical distribution. Most of the genetic diversity in the Mexican *S. guianensis* var. *guianensis* collection is represented by the genotypes from Nayarit and Veracruz.

4.2.2.2 *Stylosanthes guianensis* var. *occidentalis* Gama-López & P. Dávila var. nov. ined.

A total of 94 genotypes representing 44 specimens of the Mexican *Stylosanthes guianensis* var. *guianensis* collection of Gama-López were analysed by site-targeted PCR analysis. The genotypes were collected in the states of Jalisco (38), Michoacan (8) and Nayarit (48).

Seven site-targeted PCR markers were found to be polymorphic in length or DNA sequence among the *S. guianensis* var. *occidentalis* genotypes. The observed proportion of heterozygotes was 8.5%. Cluster analysis revealed two major groups in *S. guianensis* var. *occidentalis* reflecting the geographical localities from which the genotypes were collected, i.e. Michoacan versus Jalisco/Nayarit. The genotypes from Michoacan are genetically very similar. The genotypes from Jalisco form two subgroups that are correlated to their geographical distribution, i.e. three locations near the coast versus eight locations in the centre of Jalisco. The Nayarit collection contains both subgroups. There is no evidence of gene flow between the Michoacan and the Jalisco/Nayarit collection. The genetic variation that was observed in the Jalisco/Nayarit collection is represented by the genotypes from one location in Nayarit.

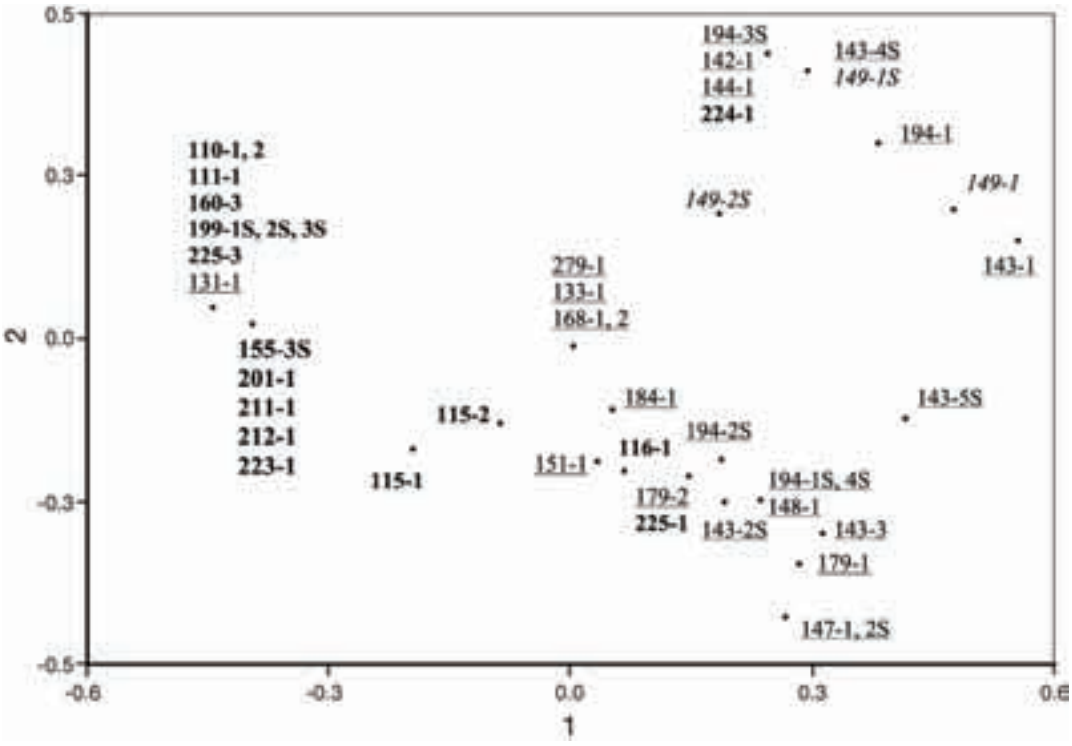


Figure 14. Two-dimensional plot based on principal coordinates analysis of similarity data from site-targeted PCR analysis, illustrating relationships among 45 genotypes of the Mexican *Stylosanthes guianensis* var. *guianensis*. Italic, double underlined, bold, underlined and waved underlined indicate the genotypes collected in Chiapas, Guerrero, Nayarit, Oaxaca and Veracruz, respectively.

4.2.2.3 *S. guianensis* var. *guianensis* ◈ *S. guianensis* var. *occidentalis* Gama-López & P. Dávila var. nov. ined.

Site-targeted PCR analysis of the *Stylosanthes guianensis* genotypes G119-2 and G257-1 with locus-specific markers always resulted in the amplification of two bands (and sometimes one heteroduplex band) that, based on their length, could be attributed to *S. guianensis* var. *guianensis* and *S. guianensis* var. *occidentalis*. Eleven site-targeted PCR markers, which were found to be polymorphic within *S. guianensis* var. *guianensis* or *S. guianensis* var. *occidentalis* (cf. Sections 4.2.2.1 and 4.2.2.2 in this publication), were used to identify the hybrid origin of these heterozygotes. The PCR markers show that the parental genome donors of these hybrids most probably occur in the same area in Nayarit where the hybrids were found.

4.2.3 *Stylosanthes dissitiflora*

Six specimens of the Mexican *Stylosanthes dissitiflora* collection, as identified by Gama-López, and seven herbarium specimens of *S. dissitiflora* were analysed by 16 PCR markers. Figure 15 shows the phenetic tree obtained from the site-targeted PCR analysis.

Five different groups were observed, each corresponding to a geographical region and morphotype. No genetic variation was observed within groups 1, 2 and 5. In contrast, small variation was found within group 4, dividing this group into two subgroups, a and b. Two heterozygotes were observed in this group. G265-1, which formed a separate group, is a hybrid, sharing one genome with *S. dissitiflora* of group 4a and one genome with *S. guianensis* var. *occidentalis*. There was no evidence of cross-hybridization between the different groups. Group 1 seems to be the most widespread *S. dissitiflora* group. Genotypes from this group were found in Honduras and in the Mexican states of Jalisco and Chiapas.

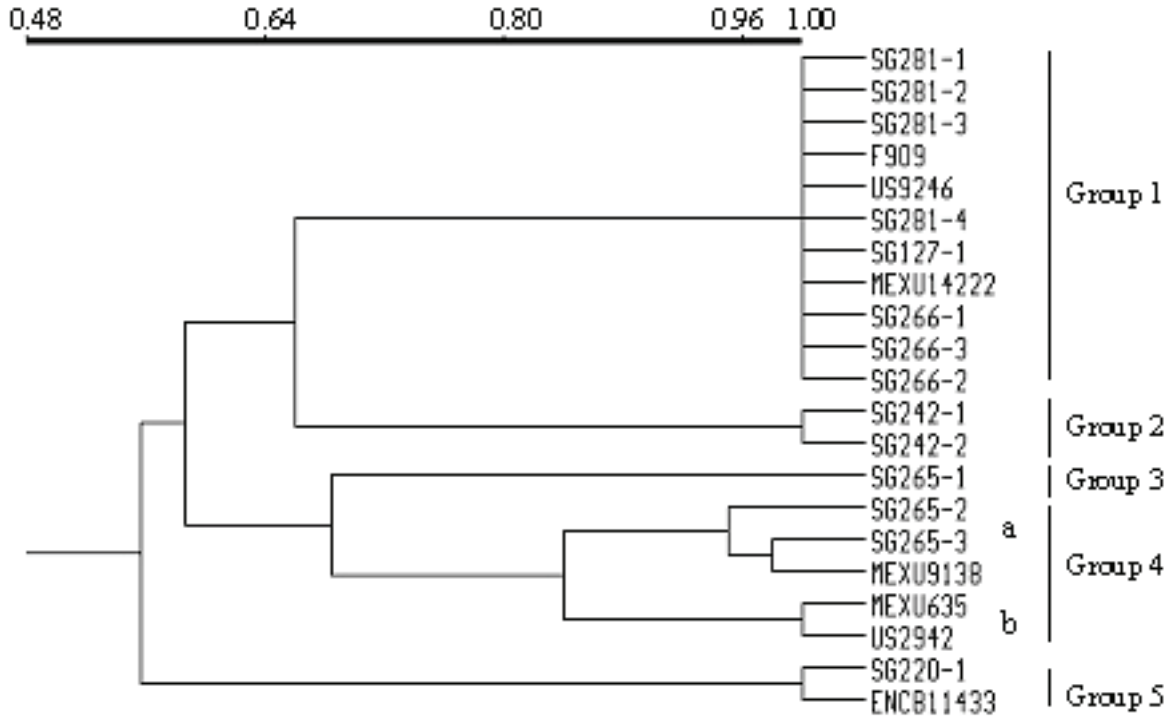


Figure 15. Phenetic tree of 21 *Stylosanthes dissitiflora* genotypes, based on similarity data from 16 site-targeted PCR markers.

4.2.4 Conclusion

From the taxonomic and molecular study it is confirmed that three different taxa in the *Stylosanthes guianensis* complex can be distinguished, i.e. *S. dissitiflora*, *S. guianensis* var.

guianensis and *S. guianensis* var. *occidentalis* Gama-López & P. Dávila var. nov. ined. Moreover, the presence of hybrids with genome additivity between these taxa suggests that they do not truly interbreed. Altogether, our data support the view of Ferreira and Costa (1979) and Costa and Ferreira (1984) that different members of *Stylosanthes guianensis* complex should be treated as different species, in contrast to 't Mannetje (1977, 1984) who regarded the taxonomic groups as different varieties. ITS DNA sequencing (cf. Section 3.3 in this publication) and site-targeted PCR analysis (Vander Stappen, unpublished data) confirmed that Mexican genotypes of *S. guianensis* var. *guianensis* are genetically similar to the genotypes of *S. guianensis* var. *guianensis* from Argentina, Bolivia, Brazil, Colombia, Guatemala, Paraguay, Peru, Suriname and Venezuela.

The considerable degree of intraspecific variation that was found within *S. guianensis* var. *guianensis* and *S. guianensis* var. *occidentalis* may be related to the breeding system. *S. guianensis* is considered to be a facultative outcrossing species (Stace 1984; Miles 1985). The observation of heterozygotes in this study gives further evidence that there exists a considerable level of outcrossing in *S. guianensis*.

4.3 Genetic diversity in Stylosanthes humilis

Plant and seed material of 30 specimens belonging to the species *Stylosanthes humilis* Kunth were collected by Gama-López in seven Mexican states, i.e. Chiapas (1), Guerrero (3), Jalisco (1), Michoacan (8), Nayarit (8), Oaxaca (8) and Veracruz (1). Difficulties in species determination were encountered in the plant material that was collected in Oaxaca and Guerrero. At these collection sites, Gama-López discovered a new species, *S. pseudohumilis* Gama-López & P. Dávila sp. nov. ined., that is morphologically similar to *S. humilis* but differs from it by the presence of an axis rudiment and two internal bracteoles and by the ploidy level. The taxonomic study of *S. humilis* by Gama-López (2006) has also shown the presence of a new ecotype of *S. humilis* in Mexico that grows in Guerrero (genotype Z415), Oaxaca (genotype G150) and Veracruz (genotype M1807) on volcanic soil and is characterized by smaller vegetative and reproductive structures in comparison with the more common *S. humilis* in Mexico. To measure the genetic diversity of *S. humilis*, molecular analysis was performed on these Mexican genotypes and on additional genotypes representing the geographical distributional range of *S. humilis*.

4.3.1 Chloroplast and nuclear DNA sequence analysis

Chloroplast DNA sequence analysis revealed the existence of two different cpDNA haplotypes A and B in *S. humilis* (Table 5). These cpDNA haplotypes are related to the geographical distribution of *S. humilis*, and may be the result of independent evolution due to geographical isolation (Vander Stappen et al. 1999a). In addition, haplotype A was also found in the cultivated genotypes of *S. humilis* from Africa and Australia. Surprisingly, haplotype A is also present in the Mexican genotypes G150 and M1807, specimens taxonomically identified as a new ecotype of *S. humilis* (Gama-López 2006).

DNA sequence analysis of the ITS region of nuclear ribosomal DNA was used to determine the level of intraspecific variation in *S. humilis*. A single ITS sequence type was found per genotype. Nine different ITS sequence types were identified in total, of which seven were found in Mexican material. Based on these differences, the genotypes are clustered in two major groups, corresponding to the cpDNA typification and supporting the spatial structure of *S. humilis*. Within group 2, two subgroups were identified. The genotype with ITS sequence type D (Z415) forms a third group that shares characters with both major groups (Table 5).

4.3.2 AFLP analysis

AFLP analysis was used to determine genetic relationships in *Stylosanthes humilis* (Vander Stappen et al. 2000). Table 5 gives an overview of the different groups that were identified by AFLP analysis. Within the Mexican collection, three major groups were recognized, each corresponding to a geographical region with low within-group and high between-group variability. There were only a few exceptions, indicating a small degree of interaction between the different areas.

4.3.3 Site-targeted PCR markers

Stylosanthes humilis was analysed by 12 site-targeted PCR markers that were previously found to be variable within this species (Vander Stappen et al. 1999b,c). The PCR markers include six microsatellites and six STS markers. For each PCR marker, only one allele was observed per genotype, indicating a low level of heterozygosity in the species *S. humilis*. Results were congruent with what was found in the previous molecular analyses.

Data derived from these PCR markers were analysed and visualized by a phenetic tree (Figure 16). The genotypes are clustered in two major groups A and B (Table 5), corresponding to the cpDNA haplotypes B and A, respectively. With the exception of the new Mexican *S. humilis* ecotype G150, all Mexican genotypes are clustered in group A, together with genotypes from Guatemala, Honduras and Costa Rica. Within this group, the clusters reflect the geographical localities from which genotypes were collected, i.e. subgroup A1: Jalisco and Michoacan; subgroup A2a: Guerrero and Oaxaca; and subgroup A2b: Nayarit (Figure 17). However, there are some exceptions, such as the genotypes G122 and G192, which are clustered in a group other than that expected from their distribution. The genotypes from Chiapas and Veracruz differ slightly from these subgroups. With the exception of the genotypes of G122, no significant differences were observed among the genotypes of Nayarit, indicating their close genetic relationship. A similar conclusion can be drawn for the genotypes from the collections from Oaxaca and Guerrero. In contrast, different alleles per locus were identified in the Michoacan collection, indicating a moderate degree of genetic variability. The Central American genotypes are grouped together with this collection. Within group B, the South American gene pool, most of the genotypes grouped according to their geographical distribution, i.e. subgroup B1a: Venezuela; subgroup B1b: Panama, Colombia; and subgroup B2: Brazil and the genotypes of Africa and Australia, which are of Brazilian origin. Two exceptions are the genotypes G150 and CPI40266-1 which originate from Mexico and Brazil, respectively. Although only a limited number of genotypes from the South American gene pool have been studied here, similar clusters have also been observed by Sawkins et al. (2001), who focused their work on this gene pool.

Table 5. *Stylosanthes humilis* genotypes studied, with their origin and classification based on different molecular markers.

Specimen	No.	Origin	cpDNA haplotype	ITS type	ITS group	AFLP group	STS/SSR group
Gama-López 283, MEXU	1	Chiapas, Mexico	B	F	2a	-	A1a
Gama-López 129, MEXU	1	Guerrero, Mexico	B	G	2a	A3	A2a
Gama-López 166, MEXU	1	Guerrero, Mexico	B	G	2a	A3	A2a
Gama-López 167, MEXU	1	Guerrero, Mexico	B	G	2a	A3	A2a
Palmer 25, MO		Guerrero, Mexico	B	F	2a	-	A2a
Zárate 415, MEXU		Guerrero, Mexico	B	D	3	-	-
Gama-López 221, MEXU	1	Jalisco, Mexico	B	E	2b	A2(-A1)	A1b
	2	Jalisco, Mexico	B	E	2b	A2(-A1)	A1b
	3	Jalisco, Mexico	B	-	-	-	A1b
	4	Jalisco, Mexico	B	-	-	-	A1b(-A2b)
CPI 86137		Jalisco, Mexico	B	E	2b	-	A1b
Gama-López 128, MEXU	1	Michoacan, Mexico	B	F	2a	A2	A1b
	2	Michoacan, Mexico	-	-	-	-	A1b
	3	Michoacan, Mexico	-	-	-	-	A1b
	4	Michoacan, Mexico	-	-	-	-	A1b

Table 5 (cont.)

Specimen	No.	Origin	cpDNA haplotype	ITS type	ITS group	AFLP group	STS/SSR group
	5	Michoacan, Mexico	-	-	-	-	A1b
	6	Michoacan, Mexico	-	-	-	-	A1b
	7	Michoacan, Mexico	-	-	-	-	A1b
	8	Michoacan, Mexico	-	-	-	-	A1a
Gama-López 171, MEXU	1	Michoacan, Mexico	B	H	2b	A2	A1a
Gama-López 172, MEXU	1	Michoacan, Mexico	B	H	2b	A2(-A1)	A1b
Gama-López 175, MEXU	1	Michoacan, Mexico	B	H	2b	A2	A1a
Gama-López 176, MEXU	1	Michoacan, Mexico	B	H	2b	A2	A1a
	2	Michoacan, Mexico	-	-	-	-	A1b
	3	Michoacan, Mexico	-	-	-	-	A1a
Gama-López 217, MEXU	1	Michoacan, Mexico	B	H	2b	A2	A1a
Gama-López 218, MEXU	1	Michoacan, Mexico	B	H	2b	A2	A1b
Gama-López 234, MEXU	1	Michoacan, Mexico	B	H	2b	A2	A1a
	2	Michoacan, Mexico	B	-	-	A2	A1a
	3	Michoacan, Mexico	B	-	-	-	A1b
	4	Michoacan, Mexico	B	-	-	-	A1b
	5	Michoacan, Mexico	B	-	-	-	A1b
	6	Michoacan, Mexico	B	-	-	-	A1a
	7	Michoacan, Mexico	B	-	-	-	A1a
	8	Michoacan, Mexico	B	-	-	-	A1a
Gama-López 117, MEXU	1	Nayarit, Mexico	B	F	2a	A1	A2b
	2	Nayarit, Mexico	B	F	2a	-	A2b
Gama-López 120, MEXU	1	Nayarit, Mexico	B	F	2a	-	-
	2	Nayarit, Mexico	B	F	2a	A1	A2b
	3	Nayarit, Mexico	B	-	-	A1	A2b
Gama-López 121, MEXU	1	Nayarit, Mexico	B	F	2a	-	-
	2	Nayarit, Mexico	B	F	2a	A1	A2b
	3	Nayarit, Mexico	B	-	-	A1	A2b
Gama-López 122, MEXU	1	Nayarit, Mexico	B	E	2b	-	A1b
	2	Nayarit, Mexico	B	E	2b	-	A1b
Gama-López 226, MEXU	1	Nayarit, Mexico	B	F	2a	A1	A2b
	2	Nayarit, Mexico	B	-	-	A1	A2b
Gama-López 227, MEXU	1	Nayarit, Mexico	B	F	2a	A1	A2b
	2	Nayarit, Mexico	B	-	-	A1	A2b
Gama-López 229, MEXU	3	Nayarit, Mexico	B	F	2a	A1	A2b
	4	Nayarit, Mexico	B	-	-	A1	A2b
Gama-López 231, MEXU	1	Nayarit, Mexico	B	F	2a	A1	A2b
	2	Nayarit, Mexico	B	F	2a	A1	A2b
Gama-López 130, MEXU	1	Oaxaca, Mexico	B	G	2a	A3	A2a
Gama-López 150, MEXU	1	Oaxaca, Mexico	A	B	1	B	B1a
	2	Oaxaca, Mexico	A	-		-	B1a
	3	Oaxaca, Mexico	A	B	1	-	B1a
	4	Oaxaca, Mexico	A	-		-	B1a
	5	Oaxaca, Mexico	A	-		-	B1a
Gama-López 180, MEXU	1	Oaxaca, Mexico	B	G	2a	A3	A2a
Gama-López 181, MEXU	1	Oaxaca, Mexico	B	G	2a	A3	A2a
Gama-López 182, MEXU	1	Oaxaca, Mexico	B	G	2a	A3	A2a
Gama-López 192, MEXU	1	Oaxaca, Mexico	B	F	2a	-	A2b
Gama-López 241, MEXU	2	Oaxaca, Mexico	B	G	2a	A3(-A2)	A2a
	5	Oaxaca, Mexico	B	-		A3(-A2)	A2a
Gama-López 271, MEXU	1	Veracruz, Mexico	B	G	2a	-	A1a
CPI 33829		Veracruz, Mexico	B	G	2a	A3(-?)	A2a
CPI 33830		Veracruz, Mexico	B	F	2a	A3(-?)	A2a
Martinez 1807, MEXU		Veracruz, Mexico	A	A	1	-	-
Heyde 4162, US		Guatemala	B	F	2a	-	A1b
Casco-Varela 32, MO		Honduras	B	F	2a	-	A1b
Standley 55048, F		Honduras	B	F	2a	-	A1b
Opler 1976, MO		Costa Rica	B	I	2a	-	A1b
MacKee 11171, US		Costa Rica	A	A	1	-	-
Mori 3687, MO		Panama	A	A	1	-	B1b
Standley 27403, US		Panama	A	A	1	-	B1b
CIAT 2334		Colombia	A	A	1	B	B1b
CPI 61674		Venezuela	A	B	1	B	B1a
CPI 40266	1	Brazil	A	A	1	B	B1b
CPI 40266	2	Brazil	A	A	1	-	B2
CPI 40266	3	Brazil	A	-	-	-	B2
CIAT 1304		Brazil	A	C	1	B	B2
Françoise Munaut 14		Côte d'Ivoire (Brazilian type)	A	A	1	B	B2
cv. Paterson		Australia (Brazilian type)	A	A	1	B	B2

4.3.4 Genetic diversity

Due to its predominantly self-pollinating nature, *S. humilis* is expected to have a low degree of outcrossing, resulting in a low degree of genetic variability. Although the levels of outcrossing in *S. humilis* are not known, failure to detect heterozygotes in this study may indicate a low level of outcrossing in this species, resulting in low levels of polymorphism within populations. Most of the genetic diversity in *S. humilis* is found between the regional types and may be associated with different habitats.

The occurrence of genetically distinct types in *S. humilis* is substantiated by additional molecular data from isozymes (Stace and Cameron 1984), RAPD (Kazan et al. 1993a,b) and AFLP analysis (Sawkins et al. 2001). The genotypes studied by Sawkins et al. (2001) could be divided into a Brazilian group, a Venezuelan/Colombian group, a Central American group (Panama, Costa Rica) and a Mexican group, with a clear discrimination of the Mexican group from the others.

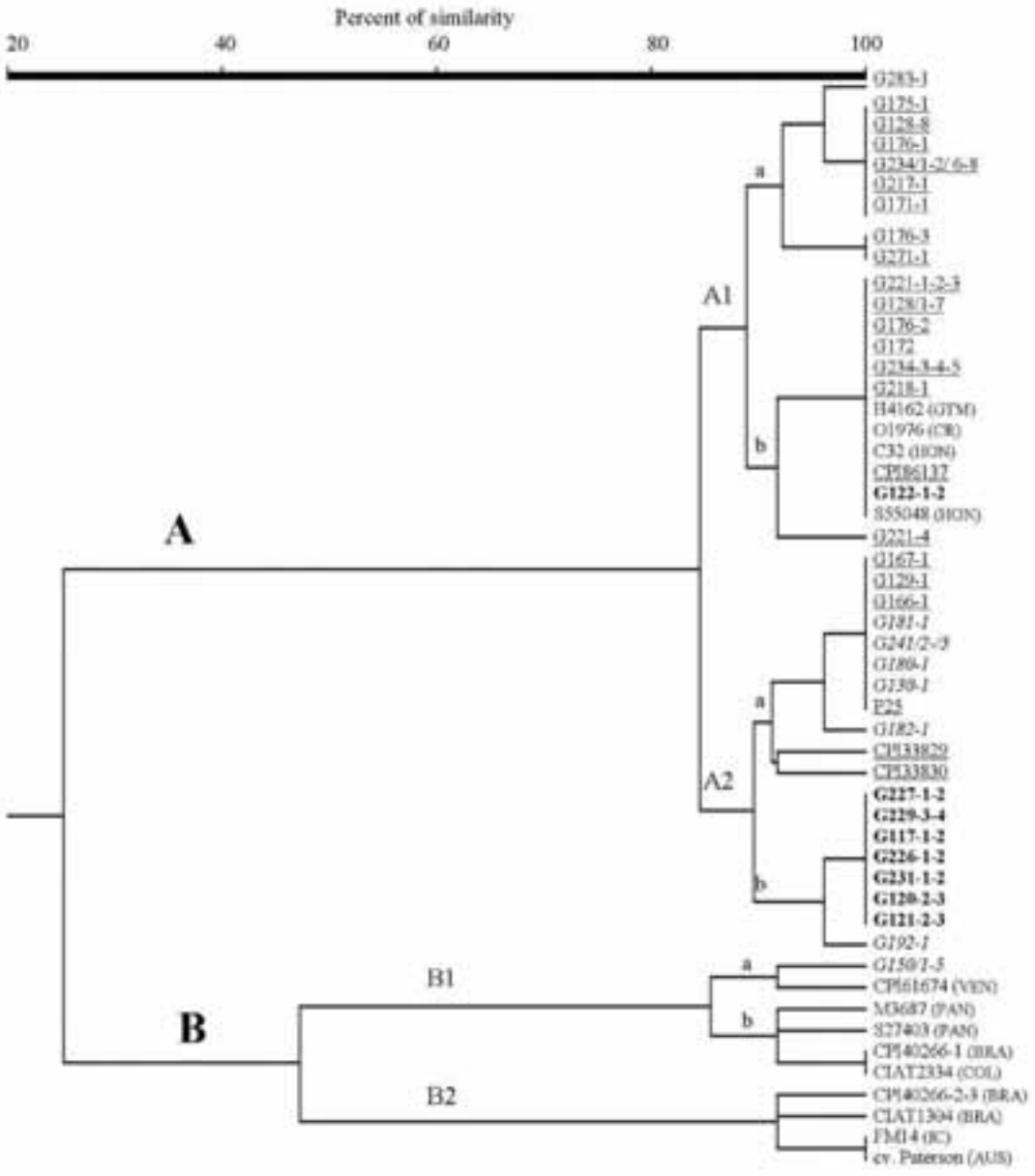


Figure 16. Phenetic tree of 78 *Stylosanthes humilis* genotypes based on similarity data from 12 site-targeted PCR markers, indicating relationships within *S. humilis*.

The clear distinction between *S. humilis* of Mexican and Brazilian origin has also been demonstrated by F_1 hybrid weakness. Stace and Cameron (1984) observed in hybridization studies that F_1 hybrids formed between a Mexican and an Australian accession of Brazilian origin were significantly less fertile than F_1 hybrids between a Brazilian and an Australian accession. Altogether, these data suggest the presence of at least two major gene pools in *S. humilis*, one extending from Mexico to the northern part of Costa Rica, and the other from Costa Rica to Brazil. Most probably, both groups may have diversified independently during evolution of the species. A similar pattern of evolution has been reported for *Phaseolus vulgaris* and *Theobroma cacao,* which both have a Mesoamerican and a South American gene pool (Koenig and Gepts 1989; Whitkus et al. 1998). The existence of partial reproductive isolations separating both groups is indicative of geographical isolation over a long period. The occurrence of distinct types within each gene pool reflects the distribution of *S. humilis* in isolated enclaves from Mexico, along the Panama isthmus, through Colombia and Venezuela, down to Brazil (Mohlenbrock 1958; Williams et al. 1984), and may indicate very limited gene flow between these regions.

Surprisingly, the Mexican specimens of the new ecotype of *S. humilis* (G150, Z415 and M1807) show closer genetic relationship with the genotypes of the South American gene pool (Table 5), and hence did not conform to this geographical delineation. A possible explanation for the occurrence of this ecotype in Mexico could be long-range dispersal events or introductions of genotypes from one area to another by human activity. However, the existence of the intermediate type Z415 that shares characteristics with both gene pools, may suggest that pockets of variability exist in Mexico that do not belong to the greater Mexican gene pool.

Figure 17. Geographical clustering of the Mexican *Stylosanthes humilis* collection as revealed by site-targeted PCR markers (1 corresponds with subgroup A1; 2 with subgroup A2a; and 3 with subgroup A2b).

4.3.5 Conclusion

This study reveals new information concerning genetic variation in *S. humilis* from Mexico, an area neglected during earlier germplasm collections of *S. humilis* (Schultze-Kraft et al. 1984). The data presented here have shown that Mexico is a possible major centre of diversity for the species *S. humilis,* which may contain unique genetic resources of the species *S. humilis*, and therefore cannot be neglected in terms of its conservation.

4.4 Genetic diversity in Stylosanthes pseudohumilis *Gama-López & P. Dávila sp. nov. ined.*

Although morphologically very similar, *Stylosanthes pseudohumilis* Gama-López & P. Dávila sp. nov. ined. differs from *S. humilis*, by the presence of an axis rudiment and two internal bracteoles, and by the ploidy level, which is tetraploid (cf. Chapter 3 in this publication). In this study, molecular analyses were performed to explore the origin, diversity and parentage of *S. pseudohumilis* in relation to its putative parental genome donors *S. humilis* and *S. hamata* s.str., and the allotetraploid race of *S. hamata* s.l.

Plant and seed material of 27 specimens belonging to the species *S. pseudohumilis* sp. nov. ined. were collected by Gama-López in 6 Mexican states, i.e. Chiapas (2), Guerrero (1), Oaxaca (20), Puebla (1), San Luís Potosí (1), Tamaulipas (1) and Veracruz (1). This new species was first identified by Gama-López in Oaxaca and Guerrero, where it grows in sympatry with the species *S. humilis*.

4.4.1 Origin and parentage

Molecular analysis is a tool to investigate the constituent genomes in *S. pseudohumilis.* A single distinct chloroplast *trn*L intron and *trn*LF intergenic spacer region (IGS) sequence type was identified previously in the specimen G136 of *S. pseudohumilis*. Based on this sequence, *S. pseudohumilis* is clustered with *S. humilis* and the tetraploid race of *S. hamata* s.l. (Vander Stappen et al. 1999a). DNA sequencing of the *trn*L intron and *trn*LF IGS region from eight additional specimens, representing the distributional range of *S. pseudohumilis* in Mexico, did not show intraspecific variation. In addition, site-targeted PCR with the *trn*LF IGS primers revealed that all the specimens of *S. pseudohumilis* used in this study, have the same *trn*LF IGS length variant that is shared with *S. humilis* of the Mexican gene pool, as opposed to *S. humilis* of the South American gene pool.

In a previous study, DNA sequencing of the entire nuclear ribosomal ITS region revealed a single distinctive ITS sequence type in the *S. pseudohumilis* specimen G136 (Vander Stappen et al. 2002a). This type was also found in the other specimens of *S. pseudohumilis* collected by Gama-López and was most similar to the two ITS sequence types that have been observed in the species *S. hamata* s.str. (types A and B) and the tetraploid race of *S. hamata* s.l. (type B).

The origin of the tetraploid *S. pseudohumilis* was further investigated by STS PCR with three selective primer pairs. These primer pairs had been used in a previous study to identify the progenitors of the species *S. quintana-roensis* Gama-López & P. Dávila sp. nov. ined. (Vander Stappen et al. 2002b). The selective primer pairs amplified one fragment in *S. pseudohumilis* that could be attributed to one of its progenitors. Direct DNA sequencing of these nuclear loci from *S. pseudohumilis* (specimen G136) revealed 100% correspondence to the sequence types of either *S. humilis* or *S. hamata* s.str. Given the allotetraploidy, the molecular affinity with *S. hamata* s.str. and *S. humilis*, *S. humilis* of the Mexican gene pool and *S. hamata* s.str. may have acted as the maternal and paternal genome donors of the species *S. pseudohumilis*, respectively.

The strong affinity of *S. pseudohumilis* to *S. humilis* is substantiated by morphological and distributional data, because both species can grow in sympatry and they share a lot of morphological characters, including the diagnostic fruit characters (Gama-López 2006). The

contribution of a second genome donor from section *Styposanthes* to *S. pseudohumilis* was observed by the presence of an axis rudiment and two inner bracteoles. The hybrid origin of *S. pseudohumilis* is in direct agreement with the evolutionary model for tetraploid formation in *Stylosanthes* (Stace and Cameron 1984) because the species is a combination of a diploid species from section *Styposanthes*, i.e. *S. hamata* s.str., with a diploid species from section *Stylosanthes*, i.e. *S. humilis*. Moreover, as in most progenitors of the *Stylosanthes* tetraploids, the parental species of *S. pseudohumilis* are members of either clade 3 or clade 1 (cf. Section 3.3 in this publication).

4.4.2 Genetic diversity

AFLP analysis indicated a low level of intraspecific variation in *S. pseudohumilis*. In addition, *S. pseudohumilis* and its putative progenitors *S. humilis* and *S. hamata* s.str. were analysed by 14 non-selective site-targeted PCR markers which previously were found to be variable within one of its putative parental genome donors (Vander Stappen et al. 1999b,c, 2002b). The PCR markers included five microsatellite and nine sequence-tagged site markers. Additivity of the PCR banding pattern in *S. pseudohumilis* was observed for 11 of the 14 site-targeted PCR markers (an example is shown as Figure 18). For each primer pair in the site-targeted PCR, all the specimens of *S. pseudohumilis* contained an identical banding pattern, of which the fragments were shared with at least one of their putative parents. Two primer pairs formed an exception by amplifying two different length fragments among the specimens. These length fragments were related to the length polymorphisms that were observed in *S. humilis* of Mexican origin.

In contrast to the morphological characters, which may vary greatly along the distributional range of *S. pseudohumilis* sp. nov. ined. (Gama-López 2006), the genetic variation within *S. pseudohumilis* sp. nov. ined. is very low when compared to genetic diversity levels determined previously in *S. humilis* by AFLP analysis (Vander Stappen et al. 2000; Sawkins et al. 2001). The lack of genetic variation in *S. pseudohumilis* in comparison to the greater polymorphism observed in its parental progenitor *S. humilis* in Mexico (Vander Stappen et al. 2000; cf. *S. humilis* in this chapter), suggests that *S. pseudohumilis* has originated from a single and relatively recent hybridization event between *S. humilis* and *S. hamata* s.str. and that the observed morphological diversity is most probably due to adaptation to different environments. Additional evidence for the recent origin is provided by the congruency of several DNA markers to their corresponding diploid orthologues.

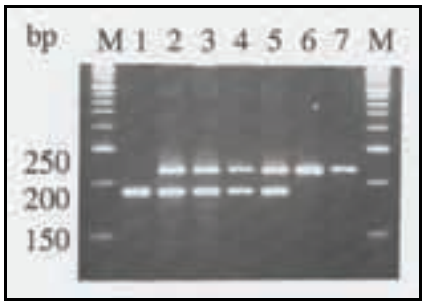


Figure 18. PCR products amplified by the STS primer pair SHCAPEAF11-R1 in the following samples: 1. *S. hamata* s.str. (2x) CPI 73523; 2. *S. hamata* s.l. (4x) Kirkbride 2475; 3. *S. hamata* s.l. (4x) Vander Stappen 33; 4. *S. pseudohumilis* Gama-López & P. Dávila sp. nov. ined. Gama-López 136; 5. *S. pseudohumilis* sp. nov. ined. Gama-López 141; 6. *S. humilis* Gama-López 180; 7. *S. humilis* Vander Stappen 17. Lane M is the DNA size marker.

4.4.3 Genetic relationship of *Stylosanthes pseudohumilis* Gama-López & P. Dávila sp. nov. ined. with the tetraploid race of *S. hamata* s.l.

The agronomically important tetraploid race of *S. hamata* s.l. (*S. hemihamata* Stace & Cameron sp. nov. ined., Stace and Cameron 1987), which is restricted to the coastal region around the Gulf of Maracaibo in Venezuela and Colombia, has an allotetraploid origin, with the diploid species *S. humilis* and *S. hamata* s.str. as its putative parental genome donors (Curtis et al. 1995; Liu et al. 1999). The AFLP banding patterns of *S. pseudohumilis* and of *S. hamata* s.l. showed great similarity to each other, with differences in absence or presence of approximately 17 bands, most of which are attributed to differences between the Mexican and Venezuelan/Colombian *S. humilis*. *S. pseudohumilis* and *S. hamata* s.l. also revealed similar STS banding patterns. Variation between both allotetraploid species were due to differences in additivity with three site-targeted PCR markers, and length polymorphisms within the diploid species *S. humilis* or *S. hamata* s.str. The length polymorphism that was observed between *S. pseudohumilis* and the tetraploid race of *S. hamata* s.l. by five site-targeted PCR markers was directly related to variation between Mexican and Venezuelan/Colombian *S. humilis*.

4.4.4 Conclusion

AFLP analysis and STS PCR markers provided molecular evidence about the close genetic relationship between *Stylosanthes pseudohumilis* Gama-López & P. Dávila sp. nov. ined. and the tetraploid race of *S. hamata* s.l. because of their common allotetraploid origin, i.e. they share the same parental species. The morphological differences between both species may be the result of independent hybridization events involving different genotypes of *S. humilis* and *S. hamata* s.str. The hybrid infertility (Stace and Cameron 1984), and the remarkable degree of morphological (Edye et al. 1974) and molecular variation (cf. Section 4.3 in this chapter) demonstrated previously between *S. humilis* of the South American and Mexican gene pools, may be reflected in the variation between the tetraploid race of *S. hamata* s.l. and *S. pseudohumilis.* This has been demonstrated by the presence of two chloroplast DNA types in *S. humilis,* enabling distinction between *S. pseudohumilis* and the tetraploid race of *S. hamata* s.l. in correlation to the geographical structuring of *S. humilis*. (Vander Stappen et al. 1999a). This was further confirmed by AFLP and five nuclear site-targeted PCR markers, where differences in PCR fragments could be assigned to differences between the two *S. humilis* gene pools. Besides differences between the maternal genome donors of *S. pseudohumilis* and the tetraploid race of *S. hamata* s.l., the intraspecific variation in the paternal genome donor may be reflected in the tetraploids. This was observed by the variation in ITS DNA sequence type, which can be directly related to *S. hamata* s.str., and to a lesser extent by polymorphism in the AFLP and STS banding pattern.

4.5 Genetic diversity of Stylosanthes viscosa

The species *Stylosanthes viscosa* is a perennial diploid species that is widespread across the Neotropics and that shows a number of different ecotypes (Costa and Ferreira 1984; Williams et al. 1984). In Mexico, this species has a wide distribution along the coasts of the Pacific Ocean and the Gulf of Mexico.

Plant and seed material of 19 specimens belonging to the species *S. viscosa* Sw. were collected by Gama-López in four Mexican states, i.e. Guerrero (2), Jalisco (1), Nayarit (13) and Oaxaca (3). Molecular analysis was performed on this Mexican collection, together with 18 additional genotypes from germplasm collections representing the wide geographical distributional range of *S. viscosa*.

4.5.1 Chloroplast and nuclear DNA sequence analysis

DNA sequence analysis of the chloroplast *trn*L intron and of the ITS region of nuclear ribosomal DNA revealed no interspecific variation in *S. viscosa.*

4.5.2 AFLP analysis

AFLP analysis with 6 AFLP primer pair combinations was carried out on 28 genotypes of the Mexican *Stylosanthes viscosa* collection and on 18 germplasm accessions of *S. viscosa* (Figure 19). The phenetic tree contains eight different clusters and clearly shows geographical clustering of the Mexican *S. viscosa* collection, i.e. Nayarit/Jalisco (group 1) versus Oaxaca (group 8) collections (Figure 20). The three subgroups within group 1 correspond to three different collection sites. Subgroup 1a contains genotypes from two locations within two kilometres of each other. In group 8, the Oaxaca group, the two genotypes of specimen G139 are clearly separated from the other genotypes of Oaxaca, which form subgroup 8a. The Mexican germplasm accessions and the accessions of South American origin clustered according to the region of origin and geographical distribution.

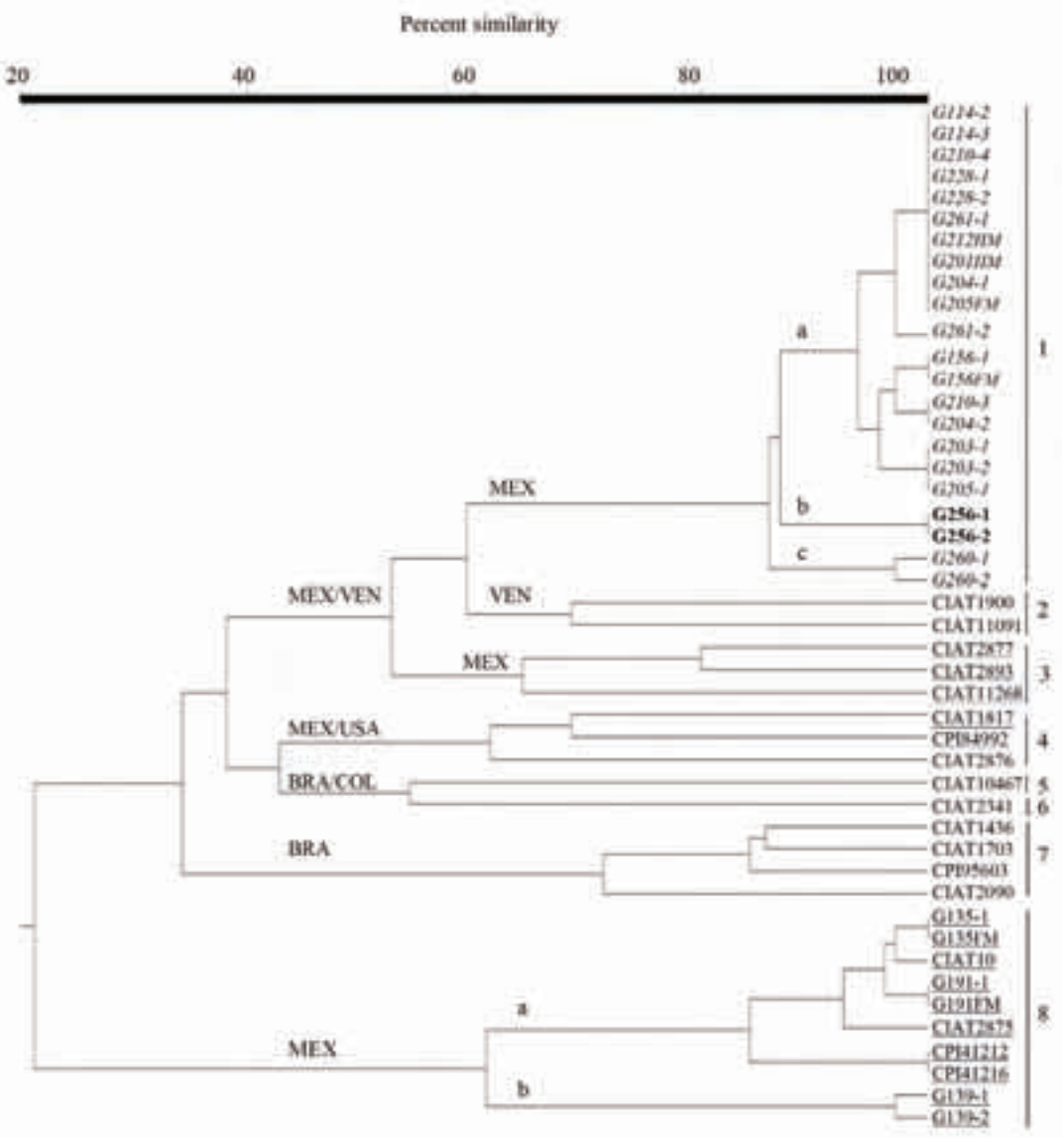


Figure 19. Representations of the AFLP data based on 70 polymorphic bands from 6 primer combinations on 46 *Stylosanthes viscosa* genotypes from Mexico (Baja California, Chiapas, Jalisco, Nayarit, Oaxaca, Tamaulipas), USA and South America (VEN: Venezuela; BRA: Brazil; COL: Colombia). The dendrogram is drawn from UPGMA cluster analysis of similarity data.

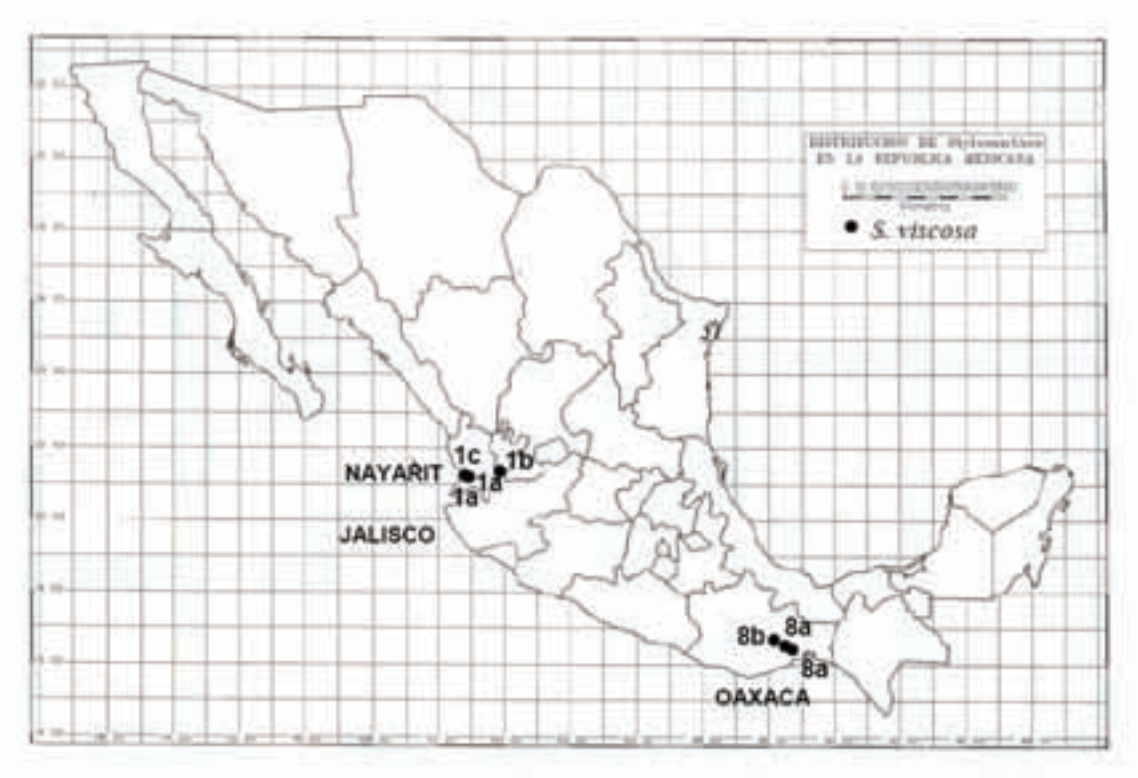


Figure 20. Geographical clustering of the Mexican *Stylosanthes viscosa* collection as revealed by AFLP analysis.

4.5.3 Site-targeted PCR markers

S. viscosa genotypes were also analysed by nine site-targeted PCR markers, previously found to be polymorphic in *S. viscosa* (Vander Stappen et al. 1999b,c, 2002b, unpublished data). The PCR markers include six microsatellite and three STS markers. The observed proportion of heterozygotes in Mexico ranged from 7.4 to 29.6%. Analysis of the data derived from these markers revealed two groups in the Mexican *S. viscosa* collection, i.e. the Nayarit/Jalisco collection and the Oaxaca collection. Within each of these groups, variation is high and heterozygotes were observed. The germplasm accessions from Baja California, Tamaulipas and Chiapas can be clearly distinguished from the Nayarit/Jalisco and Oaxaca collections by site-targeted PCR analysis, indicating that intraspecific variation in Mexican *S. viscosa* is very high and that it is related to geographical distribution. The genetic variation within the germplasm accessions from outside Mexico is also very high and heterozygotes were found here as well. Overall, the genetic variation as detected by site-targeted PCR analysis is congruent with the genetic variation as found by AFLP analysis. However, AFLP revealed a higher level of variation among the Nayarit/Jalisco genotypes.

4.5.4 Conclusion

AFLP and site-targeted PCR analysis enabled the Mexican genotypes of *S. viscosa* to be clustered into two separate groups, reflecting their geographical origin. The genetic variation that was found within these groups is much higher in comparison with the variation found in the *S. humilis* groups (cf. Section 4.3 in this chapter). These data are in agreement with the study of Sawkins et al. (2001), who suggested that *S. humilis* is genetically less variable than *S. viscosa*. The differences in genetic diversity levels between both species may be related to the breeding system in these species. *S. humilis* is considered a highly inbreeding species, whereas some evidence exists for the formation of hybrids between Mexican and Brazilian *S. viscosa,* suggesting some outcrossing in this species (Stace 1984). The observation of

heterozygotes gives evidence that there exists a considerable level of outcrossing within and between the regional groups.

In contrast to what has been observed in *S. humilis*, the genetic variation is not subdivided into a Mexican and a South American gene pool. This was also reported by Sawkins et al. (2001). Our molecular study enabled the genotypes to be clustered into a number of Mexican and Brazilian groups, and a Venezuelan, Colombian and North American group. Similar to what has been observed in the Brazilian groups (Sawkins et al. 2001), the genetic variation among the Mexican genotypes of *S. viscosa* was not continuous. This finding is very important, since *S. viscosa* is considered to be an important genome donor of allotetraploid species in *Stylosanthes* (Vander Stappen et al. 2002a; cf. Section 3.3 in this publication). In Mexico, the interaction of different forms of *S. viscosa* with a second progenitor has allowed independent evolution of similar tetraploid combinations, resulting in the formation of new species, such as *S. subsericea* and *S. tehuacanensis*, and intermediate forms, such as the tetraploid race of *S. macrocarpa*.

Given the genetic variation observed in this study and the occurrence of different ecotypes (Williams et al. 1984; cf. Chapter 3 in this publication), novel variation is likely to be present in *S. viscosa* from other locations in Mexico that have not been sampled.

5. Diversity of *Colletotrichum* species in Mexico

Most studies on anthracnose of *Stylosanthes* species have been realized on isolates from cultivated *Stylosanthes* plants, with the objective of understanding the mechanisms associated with the severe epidemics that appeared worldwide from the 1970s on. Studies on the wild pathosystem of the fungus are limited. As isolates able to overcome the resistance of newly released cultivars appear rapidly, a wide genetic diversity of *Colletotrichum gloeosporioides* is expected. For example, of the 15 cultivars that have been released in Australia between 1965 and 1996, none has remained anthracnose resistant (Chakraborty 2004). Although several experiments with the cultivars Primar and Unica, both released in 1996, had demonstrated a high level of resistance (Trevorrow et al. 1998), they had already succumbed to a new race of *C. gloeosporioides* within two years of their release (Davis et al. 1984).

Selection of resistant genotypes remains, however, the main strategy for the control of the anthracnose disease. One strategy in the search for resistance genes is evaluating simultaneously the pathogenic diversity of the fungus and that of the host, especially in areas where the plant is native and where its high genetic diversity contains the source of resistance. This research project was part of such a strategy. Diversity of the *Stylosanthes* genus was discussed in the previous chapter. This chapter focuses on the diversity of *C. gloeosporioides* and other *Colletotrichum* species infecting wild *Stylosanthes* species in Mexico. Knowledge of pathogen diversity and of host-pathogen relationships will provide useful information that will help increase targeted use of germplasm, refine collection strategies and localize pertinent areas for *in situ* conservation.

In contrast with previous studies on *C. gloeosporioides*, the isolates studied here originate from wild native *Stylosanthes* specimens collected in different states of Mexico, between 1993 and 1996. A total of 264 air-dried plant samples of different *Stylosanthes* species, collected from 78 different populations, were received from UNAM. To study the corresponding wild pathosystem of *Colletotrichum* species, each sample was evaluated for the presence of this fungus by UCL. Each isolate was coded to retain information on its origin, e.g. the code SG 199-16ac1 refers to the isolate obtained from acervulus #1 on individual plant #16 of population #199, collected by Gama-López (plant collector of UNAM).

Besides the isolates collected, African and Australian *C. gloeosporioides* isolates were also included in the experiments as reference isolates. They were previously characterized using ribosomal DNA and RAPD markers (Munaut et al. 1998) and details of previous results are given in Munaut et al. (2001).

The aim of the present work was to characterize the wild pathosystem of the pathogen on *Stylosanthes* species and to compare this diversity with artificially induced situations on the same hosts. Therefore, morphological and phylogenetic analyses were undertaken as well as an in-depth pathogenicity study.

5.1 Isolation of Colletotrichum *species*

Colletotrichum species were isolated from plant tissues showing necrotic areas, as well as from apparently healthy parts, the latter for the detection of latent infections.

During the isolation process, two main morphological types of isolates were observed, falcate- and straight-spored. A total of 72 isolates produced falcate conidia, while 198 produced straight conidia. The proportion of falcate-spored isolates from Mexican *Stylosanthes* plants was surprisingly higher than those obtained from *Stylosanthes* plants previously collected in Africa (Buxant, unpublished).

Lesions similar to the type A symptoms of *C. gloeosporioides* (for description of type A and B symptoms, cf. Section 2.2.4 in this publication) were found on 49 different *Stylosanthes* samples, collected in 27 populations in the states of Nayarit (17), Michoacan (5), Jalisco (2),

Oaxaca (2) and Veracruz (1). In the acervuli of the lesions similar to type A symptoms, straight-spored *Colletotrichum* isolates were obtained. The lesions of the *Colletotrichum* species producing falcate conidia were all associated with the greyish-brown irregular and necrotic symptoms similar, but not exactly identical, to type B anthracnose symptoms. Falcate-spored isolates originated from 19 plant samples collected from 16 *Stylosanthes* populations, of which 6 were located in the state of Oaxaca. Both straight- and falcate-spored *Colletotrichum* isolates could sometimes be observed simultaneously on the same plant (Table 6).

5.2. Colletotrichum gloeosporioides*: characterization and diversity studies*

5.2.1 Molecular identification

Recent developments in molecular genetics contributed a lot to the knowledge about the existing diversity of the studied species. As differences in gene sequence can be directly observed and described, this degree of precision was applied to investigate the evolution and taxonomy in the heterogeneous and complex *Colletotrichum* species. Molecular tools were applied to confirm the presence of *Colletotrichum gloeosporioides* in the collection of Mexican *Colletotrichum* isolates originating from wild *Stylosanthes* species. Afterwards, a preliminary study of the intraspecific diversity was carried out.

Table 6. *Stylosanthes* species and locations in Mexico where *Colletotrichum* isolates were obtained (Munaut et al. 2001).

Host (*Stylosanthes* species)	No. of plant samples	State (number of plant samples per state)
Straight-spored isolates		
Stylosanthes viscosa	18	Nayarit (18)
Stylosanthes guianensis	18	Michoacan (3), Veracruz (1), Nayarit (10), Jalisco (4)
Stylosanthes humilis	6	Michoacan (2), Nayarit (4)
SGMix (SG114, SG198, SG 199)	1	Nayarit (1)
Stylosanthes macrocarpa SG 238-3[a]	1	Oaxaca (1)
Falcate-spored isolates		
Stylosanthes viscosa	1	Oaxaca (1)
Stylosanthes guianensis	6	Nayarit (4), Michoacan (1), Jalisco (1)
Stylosanthes humilis	4	Oaxaca (2), Nayarit (2)
Stylosanthes pseudohumilis	2	Oaxaca (2)
Stylosanthes macrocarpa SG 238-1[b]/2[a]	2	Oaxaca (2)
Straight- and falcate-spored isolates obtained simultaneously		
Stylosanthes guianensis	2	Jalisco (2)
Stylosanthes humilis	1	Nayarit (1)
Stylosanthes pseudohumilis	1	Oaxaca (1)

[a] SG 238-2/3 was identified by Vander Stappen as a probable tetraploid species, with *S. viscosa* and a clade 3 species as likely progenitors (Vander Stappen, pers comm.).

[b] SG238/1 was identified by Vander Stappen as a probable hexaploid species, with as likely progenitor *S. pseudohumilis* (4x) group 1 and *S. viscosa* (Vander Stappen, pers comm.).

5.2.1.1 Amplification of a *C. gloeosporioides*-specific fragment

PCR amplifications were performed on the 270 *Colletotrichum* isolates (198 producing straight conidia and 72 producing falcate conidia), using primers synthesized on the basis of published sequences of an ITS1 fragment specific to *C. gloeosporioides* (White et al. 1990). For 120 out of the 198 straight-spored Mexican isolates, the expected target fragment was found, identifying these 120 isolates as *C. gloeosporioides*. In the remaining 78 isolates that produce straight conidia, the target fragment was not amplified. This was also the case for the 72 isolates producing falcate conidia, confirming that *C. gloeosporioides* is characterized by straight conidia (Munaut et al. 2001).

5.2.1.2 PCR-RFLP of the ITS regions

To obtain a preliminary picture of the infraspecific genetic diversity of the *C. gloeosporioides* isolates present on *Stylosanthes* species, an enzymatic restriction of the ITS1 region was performed on the 120 Mexican isolates that were identified as *C. gloeosporioides*. Four reference isolates were included in the study: an Australian reference isolate type A (HM 335) and type B (HM 336), and two African reference isolates differing from type A and B (HM 497 and HM 324). A PCR-RFLP analysis resulted in five clusters (Munaut et al. 2001) (Table 7), demonstrating intraspecific variation in *C. gloeosporioides* associated with *Stylosanthes* spp. The major cluster grouped the type A and B reference isolates together with 117 Mexican *C. gloeosporioides* isolates. The second and the fourth cluster contained only Mexican isolates, underlining the importance of diversity studies in native plant populations. The third cluster contained one African and one Mexican isolate with an identical restriction pattern. The fifth cluster contained one of the African isolates different from types A and B. The detection of isolates unrelated to type A and B at molecular level supports the necessity for an intraspecific differentiation in this highly variable species.

5.2.2 Morphological characterization

Morphological analyses allow a first rough screening of the morphotypes occurring on plants. In the case of the *Stylosanthes* anthracnose, a clear morphological distinction has been made for the isolates from cultivated areas, i.e. type A and B isolates, whilst few data are available on the wild pathosystem. It is also important to note that any description of new taxa is based on a morphological description, which can be complemented by other analyses such as a molecular characterization. Morphological characteristics as well as growth rates were studied for the four reference isolates used in for the work reported in Section 5.2.1 and for 33 Mexican *C. gloeosporioides* isolates. This restricted set of Mexican isolates was selected from the five ITS1 clusters previously described. The isolate obtained from acervulus #1 from each plant was analysed, except when this was identified as non-*gloeosporioides,* in which case it was taken from acervulus #2. Unfortunately, the strain from SG 234-5ac2 (*S. humilis*) was lost after this analysis, and could not be included in the later genetic and pathogenicity experiments. Per isolate, the length and width of 50 conidia were measured and the conidial apex shape (acute or obtuse) was recorded. After subculturing each isolate, the diameter (mm) of the six replicate subcultures was measured daily until the Petri dish was covered completely with mycelia, which allowed calculation of the daily growth rates (mm/day). After 12 days of incubation, the overall appearance of colonies, as well as the vegetative and reproductive structures and several conidial and appressorial characteristics, were described, using 32 multi-state categories (Munaut et al. 2001).

Characterization of the conidial morphology showed that the type B reference isolate produced, as expected, more conidia with acute apices (71%) than the type A reference isolate (29%). The mean percentage of conidia with acute apices produced by Mexican isolates of *S. guianensis* origin, which were clustered together with the Australian types A and B, was 82%, while that of the isolates originating from other *Stylosanthes* species was

only 55%. Mean growth rate of isolates from wild Mexican *S. guianensis* species (7.8 mm/day) was slower than the mean rate (11.9 mm/day) for isolates from other *Stylosanthes* species. Similar results were reported for type A and B isolates from cultivated *Stylosanthes* species (Irwin and Cameron 1978). This suggests that both types A and B occur in the wild and that they have a common genetic background with the type A or B isolates described on cultivated *Stylosanthes*.

The overall appearance of the colonies on PDA culture medium was very variable for all the isolates studied. An index of stability (S) was calculated for each morphological characteristic in order to evaluate its pertinence for discrimination of isolates (Munaut et al. 2001). For example, S = 100% (the highest level of pertinence) means that the colonies subcultured from an isolate presented the same state for a characteristic as the original isolate. Only 8 of the 32 morphological characteristics had a stability index of 100%. These eight characteristics were: (1) distribution of sclerotia per dish; (2) number of sclerotia per dish; (3) presence of setae in acervuli; (4) distribution of perithecia per dish; (5) number of perithecia per dish; (6) ascospores mass colour; (7) edge of the appressoria; and (8) size of the appressoria. Except for the sclerotia characteristics, the characteristics describing the overall appearance of the colonies and the mycelia were less stable (S = 13.9–58.3%) than those describing the reproductive structures (S = 72.4–100%). This high level of morphological variation, not only between colonies from different isolates, but also between strains subcultured from the same isolate, underlines the limits of morphological characterization of the variable *C. gloeosporioides* species (Munaut et al. 2001).

Based on these 32 morphological categories, the relationships between the colonies of 33 Mexican and the 4 reference isolates were evaluated using the unweighted pair group method with arithmetic averages (UPGMA). The tree generated grouped the isolates in three major clusters, I, II and III, and was partially related to the PCR-RFLP clustering of the ITS1 regions (Table 7) and to the host from which they were isolated (Munaut et al. 2001). Based on the morphological variations observed between subcultures from the same isolate for some characteristics, these subcultures can belong to different morphological clusters. The first major cluster contained colonies from the Australian isolate type A (HM 335), and from 21 Mexican isolates originating from *S. humilis* and *S. viscosa,* all characterized by an ITS1 pattern, as well as colonies from one isolate from *S. guianensis* and one from *S. guianensis,* but presenting ITS RFLP patterns at variance with type A and B reference strains (ITS clusters 2 and 4 respectively). The second major cluster also contained 12 isolates from *S. viscosa*, *S. humilis* and from 3 *S. guianensis* plants, which in this case belong to the ITS1 cluster. In the third major cluster, the Australian type B isolate (HM 336) was found together with the two isolates from Mexican *S. guianensis* (ITS cluster 1), as well as with a mix of strains from various hosts and origins, but different from type A and B reference isolates (ITS clusters 3 and 5). Similarly, a classification by Davis et al. (1992) based on morphological characteristics of *C. gloeosporioides* isolates causing type A and B symptoms worldwide also demonstrated the occurrence of three main clusters, containing heterogeneous groups of strains. According to their study a first cluster contained a mixture of type A and B isolates, a second contained mostly type A isolates, and a third cluster mostly type B isolates.

Table 7. Summary of results from molecular (based on ITS1 markers) and morphological clustering of Mexican *Colletotrichum gloeosporioides* isolates infecting *Stylosanthes* species.

Isolate code	Host species	Type of symptoms	ITS1 RFLP cluster	Morpho-logical clustering	RAPD[b]	ITS1 sequence cluster	accession in EMBL
HM 335[c]	*S. viscosa*	A	1	Ia/IIa	I1	S	Y16198
SG 114-2 ac1	*S. viscosa*	A	1	Ia	I4	S	AJ318632
SG 114-9 ac1	*S. viscosa*	A	1	Ib/IIa	I5	S	AJ318633
SG 128-1 ac1	*S. humilis*	A	1	Ia/IIa	I12	S	AJ318747
SG 156-1 ac1	*S. viscosa*	A	1	Ia/Ib/IIa	I2	S	AJ318634
SG 201-1 ac1	*S. viscosa*	A	1	Ia	I2	S	AJ318637
SG 205-1 ac1	*S. viscosa*	A	1	Ia/IIa	I2	S	AJ318638
SG 205-2 ac1	*S. viscosa*	A	1	IIa	I2	S	AJ318639
SG 210-1 ac1	*S. viscosa*	A	1	Ia	I2	S	AJ318640
SG 210-2 ac1	*S. viscosa*	A	1	Ia/Ib	I2	S	AJ318641
SG 210-3 ac1	*S. viscosa*	A	1	Ia	I2	S	AJ31863
SG 210-4 ac1	*S. viscosa*	A	1	Ia/IIa	I3	S	AJ318643
SG 210-5 ac1	*S. viscosa*	A	1	Ia	I2	S	AJ318644
SG 212-1 ac1	*S. viscosa*	A	1	Ia/IIa	I2	S	AJ318645
SG 226-2 ac1	*S. humilis*	A	1	Ia/IIa	I6	S	AJ318646
SG 226-3 ac1	*S. humilis*	A	-	(no data)	I9	S	AJ318647
SG 226-5 ac1	*S. humilis*	A	1	Ia	I6	S	AJ318648
SG 226-6 ac1	*S. humilis*	A	1	IIa	I9	S	AJ318649
SG 228-2 ac1	*S. viscosa*	A	1	Ia/IIa	I2	S	AJ318650
SG 228-3 ac1	*S. viscosa*	A	1	Ia/Ib	I7	S	AJ318651
SG 228-4 ac1	*S. viscosa*	A	1	Ia/IIa	I7	S	AJ318652
SG 228-6 ac1	*S. viscosa*	A	1	Ia	I7	S	AJ318653
SG 228-7 ac1	*S. viscosa*	A	1	Ia	I7	S	AJ318654
SG 229-5 ac1	*S. humilis*	A	1	IIa	I2	S	AJ318655
SG 231-5 ac1	*S. humilis*	A	1	Ib	I9	S	AJ318656
SG 234-5 ac1	*S. humilis*	A	1	Ib	I9	S	AJ318657
SG 234-5ac2	*S. humilis*	no data	2	Ic	no data	no data	
SG 238-3 ac1	*S. macrocarpa*		1	IIIa/IIIb	I13	S	AJ318748
HM 336[c]	*S. guianensis*	B	1	IIIa	II1	S	Y16195
SG 174-1 ac2	*S. guianensis*	B	1	IIb/IIIa	II2	S	AJ318631
SG 177-1 ac1	*S. guianensis*	B	1	II a	II3	S	AJ318749
SG 194-1 ac1	*S. guianensis*	B	1	IIIa	II6	S	AJ318635
SG 199-15 ac1	*S. guianensis*	B	1	IIa/IIb	II7	S	AJ318636
HM 497[c]	*S. guianensis*	C	3	IIIc	III1	VHO1	Y16203
SG 205-4 ac2	*S. viscosa*	C	3	IIIb/IIIc	III2	VHO2	AJ318751
HM 324[c]	*S. guianensis*	C	5	IIIb	IV1	VHO2	Y16200
SG 225-3 ac1	*S. guianensis*	C	4	Ic	IV2	VHO2	AJ18750

[a] Symptoms of type A and B induced in pathogenicity tests; C = necrotic lesions, different from either type A or B lesions.

[b] RAPD subcluster delineated at 90% similarity.

[c] Reference isolates.

5.2.3. Genetic diversity

One can say that characterization based only on morphology is not sufficient. The observed variability of morphological and cultural characteristics in combination with a complex species such as *C. gloeosporioides,* and its corresponding genus *Colletotrichum*, increases the difficulty in interpreting diversity data above inter-isolate levels. Therefore a molecular approach was needed to compare the genetic diversity and the phylogenetic relationships of the 119 *C. gloeosporioides* isolates from wild Mexican *Stylosanthes* plants with isolates from cultivated *Stylosanthes* species of other countries and from other host plants.

5.2.3.1 RAPD analysis

A total of 88 reproducible polymorphic bands were generated with six primers. The UPGMA method of clustering revealed a grouping of the 123 isolates (119 Mexican and 4 reference isolates) in 4 major polymorphic RAPD clusters (I to IV) (Table 7) (Munaut et al. 2002). Cluster I contained the Australian reference isolate type A and most of the Mexican isolates from the *S. humilis* and *S. viscosa* plants, as well as *S. macrocarpa* (SG 238-3). Within this cluster, an important level of polymorphism was observed and isolates were split into 13 subclusters. These subclusters separated the Australian reference isolate type A from the Mexican ones. Most of the isolates belonging to the same subcluster originated from the same host species. Cluster II grouped the Australian reference isolate type B and 14 isolates originating from Mexican *S. guianensis*. The level of polymorphism in this cluster was even higher than in cluster I, and nine subclusters were generated. DNA polymorphism was even detected between two isolates of the *S. guianensis* plants, collected in the same area but at two slightly different altitudes. Clusters III and IV each contained one African reference isolate and one Mexican isolate, all differing from the typical type A and B isolates (Munaut et al. 2001).

This genetic analysis showed that both type A and B are found on wild native *Stylosanthes* species in Mexico, and that within each type, the Mexican isolates were more closely related to each other than to the Australian reference isolates, suggesting a distinct genetic background of *C. gloeosporioides* populations according to their geographical origin (Munaut et al. 2002). Furthermore, the RAPD patterns of the isolates belonging to clusters III and IV differed from those of the typical type A and B isolates, determining them to be type C isolates.

5.2.3.2 Phylogenetic analysis

The important diversity in *Colletotrichum gloeosporioides* isolates, as well as the occurrence of Mexican isolates unrelated to type A and B, indicated the need to investigate the phylogeny of *C. gloeosporioides* isolates originating from *Stylosanthes* and from other hosts.

Thirty-two Mexican isolates, selected from the work described in Section 5.2.2 for morphological analysis, with the exception of the isolate SG 234-5 ac2, were sequenced together with 26 *C. gloeosporioides* isolates from *Stylosanthes* species from outside Mexico and from other hosts. The ITS1 regions of the *C. gloeosporioides* isolates were analysed with the ITS1 regions of isolates of *C. gloeosporioides* from various hosts and origins available in the European Molecular Biology Laboratory (EMBL) databank and with the ITS1 sequences of *C. fragariae*, *C. fuscum* and *C. kahawae* isolates (Munaut et al. 2002), which are all very similar to the ITS1 sequence of *C. gloeosporioides* (Sreenivasaprasad et al. 1996). *C. musae* was used as an outgroup.

Two major phylogenetic clusters, S and VHO, respectively referring to isolates mostly from *Stylosanthes* species (S) and to isolates from various hosts and origins (VHO), were generated (Table 7) (Munaut et al. 2002). Of the 30 isolates grouped within RAPD clusters I and II, and simultaneously belonging to cluster S according to their ITS1 sequence, three different ITS1 sequences (173 bp long) were identified (Table 7), slightly different from those

of the reference isolates of type A and B (Munaut et al. 2002). Cluster VHO contained the 17 isolates from various hosts and origins, as well as the isolates from *Stylosanthes* species grouped in RAPD clusters III and IV. HM 497 (RAPD subcluster III1), *C. kahawae* and the *C. gloeosporioides* isolate from *Carica papaya* subclustered separately (VHO1) from the other 14 isolates, in VHO2. Within the subcluster VHO2, isolate SG 205-4ac2 (RAPD subcluster III2) presented exactly the same sequence as reported for the isolate from *Citrus*. The isolates HM 324 and SG 225-3ac1 (RAPD subclusters IV1 and IV2) were also found in subcluster VHO2.

5.2.4 Pathogenicity

The type of symptoms induced on plants, as well as the virulence spectrum and the aggressiveness of Mexican *C. gloeosporioides* isolates were analysed to evaluate the risk of germplasm transfer to areas where *Stylosanthes* is cultivated. A selected set of 32 isolates were studied, based on their position in the RAPD analysis as described in Section 5.2.3. The isolates were inoculated on seven cultivated *Stylosanthes* species using the technique of Munaut and co-workers (1997):

- *S. viscosa* (accession DON305, S148 and S) and *S. humilis* (cv. Paterson) were chosen because they were the host species of most of the Mexican isolates.
- *S. hamata*, *S. capitata* (native in Brazil and Venezuela), *S. fruticosa* (native in Africa in semi-arid conditions) and *S. scabra* (cultivated worldwide in tropical areas) were selected as host plants because of their South American and/or tropical to semi-arid origin.
- A set of *S. guianensis* accessions and cultivars were also tested, as these were previously used to differentiate the virulence spectrum of the African and Australian type B isolates. (Malter 1984; Munaut 1990; Maraite 1993). Accessions CIAT 136, CIAT 10136, CIAT 184, CIAT 1283 and cvs. Cook, Endeavour, Graham and Schofield were tested for their susceptibility to Mexican isolates from *S. guianensis.* For Mexican isolates from other *Stylosanthes* species, only accessions CIAT 10136 and CIAT 184 and cvs. Endeavour and Schofield were tested.

In each test, the African type B isolate was used as internal control, as accession CIAT 10136 is resistant to this isolate, and accession CIAT 184 and cv. Schofield susceptible and highly susceptible, respectively (Munaut et al. 1997). The anthracnose severity index (asi) on each inoculated leaflet was recorded using a scale of: 0 = no symptoms; 1 = spots smaller than 1 mm; 2 = up to 25% of the surface necrotic; 3 = up to 50%; 4 = up to 75%; 5 = more than 75% or leaflet dropped. A mean asi was calculated for each leaf. Statistical analyses were performed using the General Linear Model of the SPSS software (SPSS, Chicago). General means (both per isolate and per inoculated genotype) were compared using the Student-Newman-Keuls test ($p<0.05$). The asi data were sorted into five categories related to the level of compatibility of the interaction: incompatibility (I), when no symptoms were observed (mean asi = 0); low compatibility (c) for 0<mean asi<1; moderate compatibility (C) for 1≤mean asi<2; high compatibility (CC) for 2≤mean asi<4; and very high compatibility (CCC) for mean asi≥4.

Most isolates originating from *Stylosanthes* species other than *S. guianensis* and clustered in RAPD cluster I induced only small type A lesions (Munaut et al. 2002) on the inoculated species. Some inoculations on *S. humilis* and *S. capitata* even resulted in incompatible interactions. Nevertheless, compatibility levels ranging from C to CC were observed in more than 50% of the interactions between the accessions S148 and S (both *S. viscosa*), and the Mexican type A isolates. No type B symptoms were observed in any of the compatible reactions (Table 8).

The four isolates belonging to RAPD cluster II were obtained from *S. guianensis*. On each *S. guianensis* genotype, except on accession CIAT 184 (susceptible to African type B isolate), extensive type B lesions were induced by at least one Mexican isolate. One isolate even demonstrated a potential to induce lesions on accession CIAT 10136, a genotype

characterized as being resistant to the African type B isolate. Only one isolate induced extensive lesions on cv. Cook. The *S. guianensis* cvs. Schofield and Endeavour were the most severely attacked. In contrast, all the genotypes of the other species were free of symptoms, or presented small spots less than 1 mm in diameter (Table 9).

Two Mexican isolates extracted from *S. viscosa* and *S. guianensis*, that differed from the typical A or B isolates at the molecular level (being respectively in RAPD clusters III and IV), induced only small, restricted necrotic lesions, different from either A or B anthracnose necrosis, on several inoculated *Stylosanthes* species (Munaut et al. 2002) (Table 8 and Table 9).

The symptoms induced on plants by Mexican isolates support the intraspecific differentiation in types A and B, as demonstrated by the RAPD results. In each compatible interaction, Mexican isolates related to type A induced type A symptoms, while isolates related to type B induced type B symptoms. Mexican type B isolates were able to induce only small, restricted lesions on various species of *Stylosanthes* other than *S. guianensis*. In contrast, a few Mexican type A isolates had the potential to induce severe type A lesions on various *S. guianensis* genotypes. This pathogenicity study proves that some isolates occurring in wild *Stylosanthes* populations were able to overcome the resistance of cultivated genotypes, constituting a major threat to the commercial utilization of *Stylosanthes* worldwide.

5.2.5 Taxonomic conclusion

The important morphological variability within type A and B isolates led to a distribution of some isolates in morphological groups containing both types. This continuum in variability has already been described for isolates from South America (Kelemu et al. 1999), and underlined again the importance of other robust criteria for taxonomic analysis of the *C. gloeosporioides* complex.

The results of the various approaches clearly demonstrated that an intraspecific level of differentiation exists within the species *C. gloeosporioides*. The present results and those obtained by various authors involved for many years in the characterization of *C. gloeosporioides* isolates from *Stylosanthes* and other hosts (Hodson et al. 1993; Alahakoon et al. 1994; Hayden et al. 1994; Sreenivasaprasad et al. 1996; Johnston and Jones 1997; Yang and Sweetingham 1998; Johnston 2000; Waller and Bridge 2000) lead to the proposal of an infraspecific taxon for the *C. gloeosporioides* isolates originating from *Stylosanthes* species, i.e. *Colletotrichum gloeosporioides* forma *stylosanthis* Munaut f. nov. anam. Within this new forma, two formae speciales were distinguished corresponding to the earlier discovered pathogenic variation in the species *C. gloeosporioides*, i.e. the type A and the type B. The corresponding formae speciales are respectively *Colletotrichum gloeosporioides* Penz. f. *stylosanthis* forma specialis *stylosanthis* Munaut f. sp. nov. and *C. gloeosporioides* Penz. f. *stylosanthis* forma specialis *guianensis* Vinijsanun, Irwin & Cameron (Vinijsanun et al. 1987). The complete taxonomical descriptions as well as the set of isolates examined can be consulted in Munaut et al. (2002).

The new forma *stylosanthis* presents the main morphological characteristics and reproductive structures of the species as described by von Arx (1957) and Sutton (1980, 1992). Some isolates (e.g. SG 199-15 ac1) occasionally produce perithecia similar to those of the broad teleomorphic species *Glomerella cingulata* (Stoneman) Spaulding & Schrenk. However, the ITS1 sequence differs significantly and consistently from the ITS1 sequences of ten isolates analysed from ten hosts other than *Stylosanthes* (96–98% similarity), while the ITS1 sequence similarity within the forma is more than 98% (Munaut et al. 2002).

The forma specialis *stylosanthis*, known as type A, is hosted on a wide range of cultivated and wild Mexican *Stylosanthes* species, but with a very low occurrence on *S. guianensis*. It induces light lesions surrounded by a dark margin and is characterized by a RAPD pattern that differs from the f. sp. *guianensis* by 70% UPGMA dissimilarity and demonstrates internal subclustering.

Table 8. Anthracnose severity on 12 genotypes of *Stylosanthes* spp. caused by inoculation of 26 isolates of *Colletotrichum gloeosporioides* from RAPD cluster I (type A isolates) and of *C. gloeosporioides* isolate SG 205-4ac2 from RAPD cluster III (type C isolate), all obtained from wild Mexican *Stylosanthes* spp. samples.

		***Stylosanthes* species**													
Host	**Isolate[a]**	***S. humilis***	***S. guianensis* CIAT184**	***S. capitata***	***S. guianensis* CIAT10136**	***S. fruticosa***	***S. scabra***	***S. hamata***	***S. guianensis* cv. Endeavour**	***S. guianensis* cv. Schofield**	***S. viscosa* DON305**	***S. viscosa* S148**	***S. viscosa* DON305**	**Mean[b]**	
S. viscosa	SG 210-5ac1	I	C	c	c	c*	c	c	I	c*	c*	c*	c*	0.19	a
S. macrocarpa	SG 238-3ac1	C	c*	c	I*	c	c	c	-	C	c	c	c	0.22	a
S. viscosa	SG 228-2ac1	I	C	c	I	I	c	I	c*	I	c	c	c	0.22	a
S. viscosa	SG 228-7ac1	I	C	I	I	c	c	c	C	C	c*	c*	C*	0.25	ab
S. humilis	SG 226-2ac1	C	I	c*	c*	c	I	c	c*	C	I	c	c	0.26	ab
S. humilis	SG 128-1ac1	C	C	c*	c	c	c	c	C	C	C	c	I	0.31	abc
S. humilis	SG 231-5ac1	C	C	c	c	c	c	c	C	C	I	c	c	0.32	abc
S. viscosa	SG 228-6ac1	I	I	c	I	c	c	I	I	C	c*	C	C*	0.32	abc
S. viscosa	SG 210-2ac1	C	C	c	I*	c	c	I	C	I	c	C	C	0.34	abcd
S. viscosa	SG 114-9ac1	C	C	c	c	I	c	c	C	C	c*	c*	c*	0.36	abcde
S. viscosa	SG 205-1ac1	C	C	I	c	c	c	c	C	C	c	c	c	0.41	abcdef
S. viscosa	SG 114-2ac1	I	C	c	c	c	I	c	C	C	c*	C*	C*	0.43	abcdef
S. viscosa	SG 228-4ac1	C	C	c	c	c	c	I	C	C	c*	c*	CC*	0.48	bcdefg
S. humilis	SG 226-6ac1	I	I	c	I	c	c	I	C	C	c	C	CC	0.49	bcdefg
S. viscosa	SG 210-4ac1	c	c*	I*	I*	C	c	c	C	I	c	C	C	0.49	bcdefg
S. viscosa	SG 201-1ac1	I*	C	c	c	c*	c	c	I	I	c	CC	CC	0.52	cdefg
S. viscosa	SG 205-2ac1	I	I*	I*	c	c*	c	c	C	C	c	C	CC	0.52	cdefg
S. humilis	SG 229-5ac1	c*	c*	c*	c*	c*	c*	c*	c*	c*	c*	C*	C*	0.53	cdefg
S. humilis	SG 226-5ac1	I	C	c	I	c	c	I	C	I	C	CC	CC	0.59	defgh
S. viscosa	SG 228-3ac1	I	I	c*	c	I	c	c	C	C	c*	C*	CC*	0.61	efgh
S. humilis	SG 234-5ac1	I	C	I	I	c	c	I	I	C	c	C	CC	0.63	fgh
S. humilis	SG 226-3ac1	c	C	c	c	c	I	c	C	C	C	C*	CC	0.68	gh
S. viscosa	SG 210-3ac1	c	c*	c	c*	c	c	c	c*	c*	c	CC*	CC*	0.69	gh
S. viscosa	SG 156-1ac1	I	I*	I*	C*	I*	c	c	C	I	C	CC	CC	0.81	hi
S. viscosa	SG 212-1ac1	I	c*	c	c	I	c	c	CC	CC	c	CC	c	0.81	hi
S. viscosa	SG 210-1ac1	c	I*	c	c	c	c*	CC	C	CC*	C	CC	CC*	0.94	i
Mean[b]		0.06 a	0.08 ab	0.13 abc	0.21 bcd	0.23 cd	0.29 de	0.29 de	0.31 de	0.41 e	0.62 f	1.33 g	1.76 h		
S. viscosa	SG 205-4ac2	c	C	c	c	c	c	c	C	C	c	c	c	0.33	

[a] Isolates belonged to the ITS1 cluster S, apart from SG 205-4 (cluster VHO).

[b] Mean anthracnose severity index (asi) in a column or in a row followed by the same letter are not significantly different according to the Student-Newman-Keuls test ($p<0.05$). Isolates and genotypes classified according to increasing mean asi. For an explanation of asi, see Section 5.2.4.

*Inoculation test repeated and giving the same level of compatibility as the one presented in this table.

Key to categories: I = Incompatibility, when no symptoms were observed (mean asi = 0); c = low compatibility for 0<mean asi<1; C = moderate compatibility for 1≤mean asi<2; CC = high compatibility for 2≤mean asi<4; and CCC = very high compatibility for mean asi≥4.

Table 9. Anthracnose severity on 16 genotypes of *Stylosanthes* spp. caused by inoculation of four isolates of *Colletotrichum gloeosporioides* from RAPD cluster II (type B) and of *C. gloeosporioides* isolate SG 225-3 from RAPD cluster IV, all obtained from wild Mexican *Stylosanthes guianensis.*

	***Stylosanthes* species**																	
Isolate[a]	***S. fruticosa***	***S. scabra***	***S. humilis***	***S. viscosa* cv. S**	***S. viscosa* DON305**	***S. viscosa* S148**	***S. guianensis* CIAT 184**	***S. hamata***	***S. capitata***	***S. guianensis* cv. Graham**	***S. guianensis* CIAT 1283**	***S. guianensis* CIAT 10136**	***S. guianensis* cv. Cook**	***S. guianensis* CIAT 136**	***S. guianensis* cv. Schofield**	***S. guianensis* cv. Endeavour**	**Mean**[b]	
SG 177-1	I	c*	C	C	c	c	c*	I	c	c	c	c*	c	c	c	C	0.29	a
SG 174-1	I	c	I	I	I	I	I	c	c	C	c	I*	c*	c*	C	CC	0.42	b
SG 194-1	c	I	C	I	I	I	c*	I	c*	c	c	c	c	C	CCC	CCC*	0.75	c
SG 199-15	I	c	I	I	I	c	c*	c	c	c	C	C	C	C	C	CC	0.8	c
Mean[b]	0.02	0.04	0.06	0.08	0.08	0.10	0.12	0.13	0.27	0.50	0.70	0.70	0.81	0.87	1.76	2.82	0.1	
	a	a	A	a	a	a	a	a	ab	bc	cd	cd	cd	d	e	f		
SG 225-3	c	c	I	C	c	c	c	c	I	c	I	c	c	c	c	I		

[a] Isolates all originating from ac1 and belonged to the ITS1 cluster S, apart from SG 225-3 (cluster VHO).

[b] Mean anthracnose severity index (asi) in a column or in a row followed by the same letter are not significantly different according to the Student-Newman-Keuls test ($p<0.05$). Isolates and genotypes classified according to increasing mean asi. For an explanation of asi, see Section 5.2.4.

* inoculation test repeated and giving the same level of compatibility as the one presented in this table.

Key to categories: I = Incompatibility, when no symptoms were observed (mean asi = 0); c = low compatibility for 0<mean asi<1; C = moderate compatibility for 1≤mean asi<2; CC = high compatibility for 2≤mean asi<4; and CCC = very high compatibility for mean asi≥4.

The forma specialis *guianensis*, known as type B, is hosted on cultivated *S. guianensis* in Australia and Africa or on wild specimens in Mexico, and on a restricted set of other wild Mexican *Stylosanthes* species (*S. gracilis*, *S. grandifolia*). It induces extensive, brown lesions and is characterized by a RAPD pattern that differs from the f. sp. *stylosanthis* by 70% UPGMA dissimilarity and demonstrates internal differentiation into clusters with more than 55% similarity. Both formae speciales are distributed in tropical and subtropical areas of Africa, Australia, Asia and Central and South America.

5.3 Straight-spored versus falcate-spored Colletotrichum *species*

The diversity aspect of the falcate and straight conidia produced by *Colletotrichum* isolates was not reported in previous work of South America, although the fungus species is a well-studied pathogen in South America.

Of the 270 *Colletotrichum* isolates retrieved from wild *Stylosanthes* species in Mexico, 198 were characterized by straight conidia and 72 by falcate. The molecular, morphological and pathogenic studies revealed a remarkable diversity within the 120 straight-spored *C. gloeosporioides* isolates. To complete the global view of the *Colletotrichum* species associated with *Stylosanthes* species in Mexico, and more particularly of their possible pathogenicity, the remaining 78 straight-spored and 72 falcate-spored *Colletotrichum* isolates were examined.

5.3.1 Straight-spored isolates

The morphology, phylogeny and pathogenicity of straight-spored isolates of *Colletotrichum* species other than *C. gloeosporioides* were analysed on a subset of 18 accessions obtained from ten populations of *S. guianensis*, one of *S. pseudohumilis*, one of *S. viscosa* and from a mixture of plants collected in different populations (Munaut 1999).

5.3.1.1 Morphological characterization

For the morphological characterization of the straight-spored *Colletotrichum* isolates, the same methodology was used as explained in Section 5.2.2. The isolates were obtained from acervuli that were characterized by brown septate setae producing pale salmon masses of conidia. Five isolates produced only conidia with obtuse apices; no conidia with acute apices were obtained. The mean length of the straight conidia ranged from 13.1 to 24.4 µm, and the mean width from 3.2 to 7.4 µm. Three of them were characterized by, simultaneously, mean conidial length (>22 µm) and width (>6 µm) significantly larger than those of most of the other isolates (the "giant" conidia). For most of the monoconidial isolates, the subcultured colonies presented variable morphology concerning colour and mycelium type (Munaut 1999).

5.3.1.2 Phylogenetic analysis

RAPD analysis. Given the visual observation of the amplified patterns, five major groups (RAPD I to V) could be distinguished (Munaut 1999). Group I contained three isolates producing conidia larger than the average, as well as one isolate that produced conidia similar in size to various isolates from the other groups. RAPD cluster II contained 11 isolates characterized by a wide range of different morphological and conidial characteristics. Clusters III, IV and V contained one isolate each.

ITS Sequencing. Given the results of the RAPD clustering, a restricted set of 13 isolates from 13 different plants collected in 12 *Stylosanthes* populations was used for ITS1 sequencing. Additionally 26 different *Colletotrichum* species from various hosts and origins available in the EMBL databank were included. *Epichloe* sp. was included as an outgroup (Munaut 1999) (Figure 21).

A restricted set of 13 isolates was selected on the basis of the morphological results for the ITS 1 analysis. Four main types (1 to 4) of sequences were detected, and partially

corresponded with the 5 RAPD clusters (Munaut et al. 2002) as follows:

- ITS1 type 1: three Mexican isolates producing "giant" conidia and the isolate SG205-4ac1 shared more than 99% similarity with an isolate of *C. crassipes* (EMBL acc. AJ56918). The four isolates presented similar RAPD patterns (Group I).
- ITS1 type 2: the same six isolates were also clustered in RAPD group II and presented some homology with various *Colletotrichum* species (*C. orbiculare*, *C. trifolii*, *C. lindemuthianum*, *C. acutatum* and *C. lupini*).
- ITS1 type 3: the two Mexican isolates belonging to this type 3 belonged to RAPD group III and IV. They presented a high similarity with isolates of the recently described new species *C. boninense* (Moriwaki et al. 2003).
- ITS1 type 4: the only Mexican isolate of this group (RAPD group V) presented 100% similarity with *C. coccodes* (Z32931).

This phylogenetic tree highlighted the significant diversity of *Colletotrichum* in Mexico. Although two isolates are very similar to *C. boninense* and another other isolate is related to *C. coccodes* they could not be identified as known species. Whether these isolates belong to a species for which no ITS1 sequence is available in EMBL, or to a species incompletely described by Sutton (1980; 1992) or von Arx (1957), or belong to an undescribed species remains unresolved. As a consequence, analyses of ultrastructural morphological characteristics, as well as of the ITS1 region of species for which the ITS1 sequence was not available, and of other regions of the genome able to discriminate species, such as the ITS2 region or the subunits of the ribosomal DNA (rDNA), should be performed before they are possibly described as a new species. Morphological analysis of the anamorphic form, observed for the three isolates, may also provide additional information on their taxonomic identification.

5.3.1.3 Pathogenicity

To know the pathogenic potential of the Mexican non-*gloeosporioides* straight-spored *Colletotrichum* isolates, these were inoculated on seven *Stylosanthes* species: *S. humilis* (cv. Paterson), *S. mucronata*, *S. capitata* accession CIAT 1405, *S. hamata* (cv. Verano), *S. scabra* (cv. Fitzroy), *S. viscosa* (S148) and *S. guianensis*. Three genotypes of *S. guianensis* analysed previously and presenting a range of susceptibility to African isolates (Malter 1984; Munaut 1990; Munaut et al. 1997; Munaut and Maraite 1998) were used as internal controls according to the description in Section 5.2.4. Inoculation was performed as per Munaut et al. (1997). In some interactions, the symptoms were different from the typical type A and B symptoms but more or less similar to the type C symptoms caused by several *C. gloeosporioides* isolates described in Section 5.2.4.

A 2-way analysis of variance (ANOVA) revealed a significant effect of the main factors 'isolate' and 'genotype' on the anthracnose severity indices (cf. Section 5.2.4 in this publication). The mean anthracnose severity indices ranged from 0.09 to 0.75 (Table 10). Compared to the other inoculated genotypes and species, *S. humilis* cv. Paterson and *S. guianensis* cv. Endeavour were respectively the most resistant and the most susceptible cultivars. Isolates induced only small, restricted lesions (c) or even no lesions (I) on *S. humilis*, *S. guianensis* accession CIAT 184, *S. fruticosa* and *S. hamata*. *S. scabra* presented extensive anthracnose lesions after inoculation with four isolates, but was free of symptoms with six others. The severe anthracnose lesions on interactions with *S. capitata*, S. *viscosa*, *S. scabra* and *S. guianensis* cv. Schofield reveals a huge pathogenic potential in *Colletotrichum* in Mexico. The ability of one Mexican isolate from *S. guianensis* and one from *S. viscosa* to induce severe lesions on *S. guianensis* accession CIAT 10136, characterized as resistant or immune to most *C. gloeosporioides* isolates from Africa (Munaut 1990; Maraite 1993; Munaut et al. 1998), confirms the potential of anthracnose disease in wild *Colletotrichum* species.

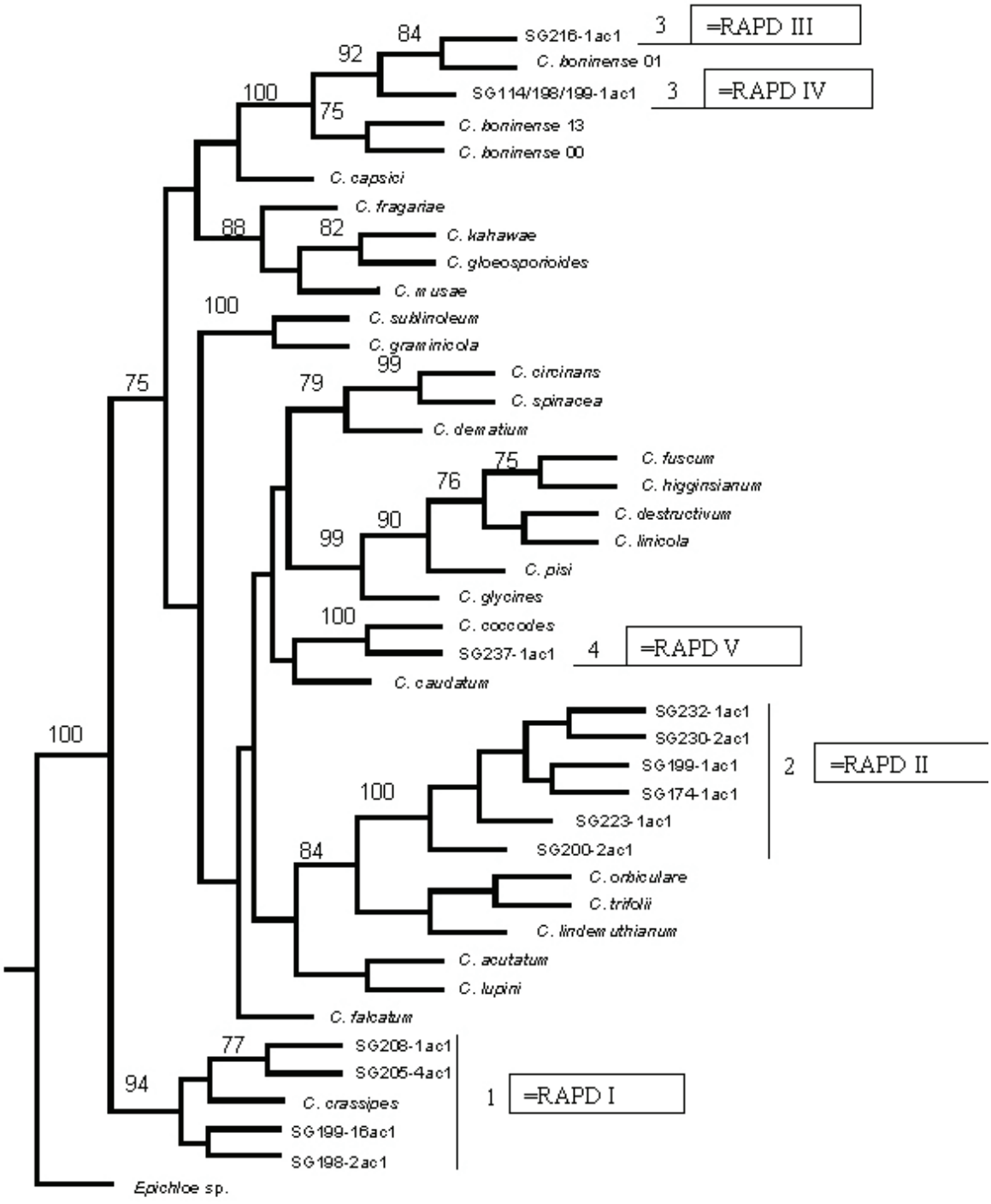


Figure 21. Phylogenetic relationships among 13 Mexican isolates of *Colletotrichum* species from *Stylosanthes* spp. and 26 species from various hosts and origins, using *Epichloe* sp. as outgroup. Consensus tree derived from parsimony analysis of 100 bootstrapped data sets of the ITS1 sequences. Number on branches indicate bootstrap values (%) from 100 replicates.

Table 10. Anthracnose severity on 10 genotypes of *Stylosanthes* spp. caused by inoculation of 18 isolates of *Colletotrichum* spp. obtained from wild Mexican *Stylosanthes* species.

	***Stylosanthes* species**											
	***S. humilis* cv. Paterson**	***S. guianensis* CIAT184**	***S. mucronata***	***S. capitata* CIAT 1405**	***S. viscosa* S148**	***S. scabra* cv. Fitzroy**	***S. guianensis* cv. Schofield**	***S. hamata* cv. Verano**	***S. guianensis* CIAT10136**	***S. guianensis* cv. Endeavour**	**Mean[b]**	
Isolates obtained from *S. guianensis*												
SG 209-5ac1	I	C	c	I	c	I	c	c	I	I	0.09	a
SG 223-5ac1	I	I	-	c	c	c	I	c	I	I	0.09	a
SG 223-4ac1	I	I	-	c	I	c	I	c	I	c	0.18	ab
SG 114/198/199[a]ac1	C	C	c	c	c	I	c	c	c	c	0.26	abc
SG 232-1ac1	I	I	c	c	c	c	c	c	c	CC	0.32	abad
SG 223-3ac1	C	C	c	c	c	c	I	c	c	c	0.33	abcde
SG 200-2ac1	I	C	c	c	c	I	c	c	c	C	0.34	abcde
SG 198-2ac1	C	C	c	I	c	I	c	c	c	C	0.39	bcde
SG 230-2ac1	I	C	c	c	I	c	c	c	c	c	0.40	bcde
SG 223-1ac1	C	C	c	c	I	CC	c	c	c	c	0.47	cde
SG 223-2ac1	C	C	c	c	c	c	c	c	c	c	0.52	chef
SG 208-1ac1	C	I	c	c	C	c	C	c	c	C	0.53	chef
SG 199-16ac1	I	C	c	I	C	I	c	c	c	C	0.54	chef
SG 216-1ac1	C	C	c	C	c	C	c	c	c	c	0.56	def
SG 199-1ac1	C	C	c	c	c	C	c	c	c	CC	0.56	def
SG 174-1ac1	I	C	c	C	c	I	CC	c	C	CC	0.75	f
Isolates obtained from *S. pseudohumilis*												
SG 237-1ac1	C	-	-	c	c	c	c	I	I	c	0.14	ab
Isolates obtained from *S. viscosa*												
SG 205-4ac1	I	C	I	I	c	C	c	c	CC	c	0.62	ef
Mean[b]	0.15	0.21	0.22	0.37	0.37	0.45	0.50	0.52	0.54	0.97		
	a	ab	ab	bc	bc	c	c	c	c	d		

[a] Isolate from a mixture of plants from the populations SG 114, SG 198, SG 199.

[b] Means in the last row and column followed by the same letter are not significantly different according to the Student-Newman-Keuls test ($P<0.05$). Isolates and genotypes classified according to increasing mean indices.

Key to categories: I = Incompatibility, when no symptoms were observed (mean asi = 0); c = low compatibility for 0<mean asi<1; C = moderate compatibility for 1≤mean asi<2; CC = high compatibility for 2≤mean asi<4; and CCC = very high compatibility for mean asi≥4. For an explanation of asi, see Section 5.2.4.

5.3.2 Falcate-spored isolates

A detailed study of several falcate-spored isolates was effected by Javaux (1998).

5.3.2.1 Morphology

Morphology of the falcate-spored *Colletotrichum* isolates was studied as described in Section 5.2.2. Within the falcate-spored isolates, two main morphological types were observed: (i) light brown colonies, producing an orange-brown pigment in the agar with very short white mycelium and absent or very sparse setae; and (ii) grey to olive-coloured colonies producing a moderately to elevated white mycelium and acervuli with seta. The isolates produced 100% of falcate conidia with acute apices. The mean size of the condia ranged from 20.9 to 28.4 µm in length and 2.5 to 2.9 µm in width.

5.3.2.2 Phylogeny

ITS, and recently obtained 28S rDNA and β-tubuline gene sequences (data not shown), revealed that most falcate-spored isolates were *C. capsici*, known as an important pathogen in tropical areas, although the homology between reference and Mexican isolates was not always perfect. Only one isolate (from *S. guianensis* plant SG 197-2) was identified as *C. dematium*.

5.3.2.3 Pathogenicity

Thirteen falcate-spored isolates that have been morphologically and molecularly identified as *C. capsici* were inoculated on three *Stylosanthes* species from which they were isolated in Mexico, i.e. *S. guianensis*, *S. pseudohumilis* and *S. humilis*. Inoculation was performed using the protocols of Munaut and co-workers (1997). Although the mean anthracnose severity indices (asi) were less than 1, except for the SG 226-1ac1 isolate, severe symptoms (C or CC) were observed in interactions with eight isolates (Table 11). *S. humilis* cv. Paterson, the most resistant genotype to Mexican *C. gloeosporioides* f. *stylosanthis* f. sp. *stylosanthis* isolates (Munaut et al. 2002), also presented the highest resistance level to isolates tested in this component of the research. Equal to the straight-spored non-*gloeosporioides* isolates, the *C. capsici* isolates also have significant pathogenic potential, as some isolates were able to induce severe lesions on *S. guianensis* accession CIAT 10136, otherwise characterized as a resistant genotype.

Table 11. Anthracnose severity on 4 genotypes of *Stylosanthes* spp. caused by inoculation of 13 isolates of *Colletotrichum capsici* obtained from wild native Mexican *Stylosanthes guianensis.*

Isolate	***Stylosanthes* species**				
	***S. hamata* cv. Verano**	***S. humilis* cv. Paterson**	***S. humilis* CIAT 10136**	***S. guianensis* cv. Schofield**	**Mean**
SG226-2ac1	CC	CC	C	I	1.9
SG241-1ac1	I	I	CC	C	0.9
SG223-4ac1	I	I	C	C	0.8
SG227-3ac1	c	I	C	C	0.8
SG183-1ac1	C	c	c	c	0.6
SG191-1ac1	c	c	c	c	0.6
SG237-5ac1	I	I	c	CC	0.6
SG238-1ac1	I	I	c	C	0.4
SG238-2ac1	I	c	I	C	0.4
SG237-1ac1	I	I	c	c	0.3
SG229-3ac1	c	c	c	c	0.3
SG237-2ac1	c	I	c	c	0.2
SG199-2ac1	c	c	I	c	0.1
Mean	0.4	0.4	0.7	0.8	

5.3.3 Consistency of morphological, molecular and pathogenicity studies

Although morphological and molecular studies are supposed to be complementary, some straight-spored *Colletotrichum* isolates of non-*gloeosporioides* species still could not be satisfactorily identified.

Four Mexican *Colletotrichum* isolates from *Stylosanthes* spp. of the ITS1 type 1 (RAPD I) that clustered with *C. crassipes*, were separated from the nine other Mexican isolates and from 26 species from various hosts and origins (Figure 21). The ITS1 sequences were the most similar to the partial sequence available for a *C. crassipes* isolate in the EMBL databank. Three Mexican isolates share some characteristics with *C. crassipes* as described by von Arx (1957), such as conidial size and shape, but the shape of the appressoria was different (Sutton 1980). Integration of the morphological and the molecular characteristics does not allow one to assign these four Mexican isolates to a known species, but gives an indication of their close proximity to *C. crassipes*. Confirmation of the species name could be validated by additional sequencing and analyses of DNA regions pertinent at the taxonomic level, such as 28S rDNA, β-tubuline and mtDNA.

Six unidentified straight-spored isolates of the ITS1 type 2 (RAPD II) were grouped into a major cluster whereby their ITS1 sequences presented a similarity rate of 94 to 98%, but these seemed to be completely different from those of the other species (Figure 21). This suggests the occurrence of a new species, characterized by small variations in its ITS1 region.

These examples suggest the occurrence of new species among the *Colletotrichum* isolates producing straight conidia. This should be clarified by comparison with isolates from various international collections, more particularly with isolates typifying species, varieties and formae of *Colletotrichum*, and with isolates for which complete morphological description or molecular data are available. Additional regions of the genome used as taxonomic tools, such as the ITS2 region or the conserved rDNA subunits (5.8S, 16S, 28S) or the β-tubuline gene, should be sequenced to evaluate the similarities of Mexican sequences with those available in the databanks.

5.4 Conclusions from the diversity study of Colletotrichum *species in Mexico*

5.4.1 No correlation between ecogeographical origin and occurrence

Although an significant number of plants (264) was collected in 78 different populations during different seasons and in a wide range of ecogeographical regions, *Colletotrichum* species could only be retrieved from 64 plants (24%) belonging to 36 populations (47%). Most of the isolates originated from plants collected during the rainy season (October to January) in the states of Nayarit, Oaxaca and Michoacan. However, correlation between ecogeographical origins of *Stylosanthes* populations and occurrence of *Colletotrichum* species is difficult to state, because of the heterogeneity in the number of collections visited in each area, in the number of plants collected within each population, and in the differences in recovering *Colletotrichum* isolates from the plants.

5.4.2 'Natural' type A and type B

Conidial morphology, growth rates on solid medium and molecular characterization all demonstrated the occurrence of both types A and B in nature, indicating a common genetic background of the isolates pathogenic on native and cultivated *Stylosanthes*.

5.4.3 High diversity

Simultaneous occurrence of *C. gloeosporioides* and other straight- and falcate-spored isolates of the *Colletotrichum* genus on native *Stylosanthes* species suggests significant diversity of the pathogen present on this host plant. Furthermore, besides the presence of type A and type B

isolates of the species *C. gloeosporioides*, confirmed through molecular and morphological characterization, some additional isolates were detected that could not be associated with type A and B isolates. The existence of *Colletotrichum* isolates sharing common molecular characteristics with, but not related to, known *Colletotrichum* taxa, and not satisfactorily identifiable using the morphological characterizations proposed by Sutton (1980) and von Arx (1957), suggests, at least, high diversity in the genus *Colletotrichum* on wild *Stylosanthes* in Mexico, and, presumably, the occurrence of new species. As expected, *Colletotrichum* isolates found on wild *Stylosanthes* species in Mexico showed higher genetic diversity than those found on cultivated *Stylosanthes*.

5.4.4 Need for an infraspecific differentiation

The integration of morphological, pathogenic and molecular data demonstrated clearly that an infraspecific level of differentiation exists within the diverse species of *C. gloeosporioides*, and that the isolates isolated from *Stylosanthes* species share a common genetic basis. Therefore the infraspecific taxon *Colletotrichum gloeosporioides* forma *stylosanthis* f. nov. was proposed for *C. gloeosporioides* isolates from *Stylosanthes*. Two formae speciales were distinguished: f. sp. *stylosanthis*, proposed for the type A isolates and f. sp. *guianensis* for the type B isolates.

5.4.5 Pathogenic potential

High genetic diversity of the pathogen and the potential for transfer of genetic information between isolates facilitates the introduction of new isolates in areas where *Stylosanthes* is cultivated. Besides that, several Mexican isolates were able to induce lesions on genotypes resistant or immune to African isolates, and therefore to put at risk commercial utilization of *Stylosanthes* worldwide. Finally, the ability of various isolates to induce anthracnose lesions on several *Stylosanthes* species demonstrated that the anthracnose epidemics worldwide reflect only a small part of the pathogenic potential in the genus *Colletotrichum*.

6. Relationship between host and pathogen diversity

The number of *Stylosanthes* species and cultivars grown worldwide is only a small part of the total biodiversity existing in the genus. This implies that interesting genes for improvement of yield, or for resistance or tolerance to various eco-climatic conditions or diseases are still supposed to be available in wild populations.

Related to this huge diversity of wild *Stylosanthes* species is the question of the occurrence and diversity of its main pathogen (*Colletotrichum gloeosporioides*) in similar wild conditions. Indeed, its presence and—even more importantly—its potential diversity, as described in the previous chapter, could be evidence that the epidemics that have occurred up till now reflect only a small part of the infection potential present in the *Colletotrichum* genus.

During this research project, huge genetic polymorphism was revealed in the *Colletotrichum* genus isolated from various wild Mexican *Stylosanthes* species. Besides the high variability detected within the 120 strains identified as *C. gloeosporioides*, the existence of some new species among the 150 other *Colletotrichum* isolates is suggested. To detect a specific relationship between the *Colletotrichum gloeosporioides* and the *Stylosanthes* species from which they originated, ITS sequencing and RAPD analyses were performed on the 120 Mexican *C. gloeosporioides* strains. Well characterized type A and B isolates from Australia were included in each analysis as reference isolates, as well as two isolates from Africa, different from both type A and B.

Sequencing of the ITS regions and phylogenetic analyses, including isolates from various other non-*Stylosanthes* hosts, demonstrated a common genomic basis for the isolates specific to the *Stylosanthes* genus. Therefore a new forma was proposed: *Colletotrichum gloeosporioides* forma *stylosanthis* Munaut F. nov. anam., in which two formae speciales were described: *C. gloeosporioides* Penz. f. *stylosanthis* forma specialis *guianensis* Vinijsanun, Irwin & Cameron mainly found on *S. guianensis;* and *Colletotrichum gloeosporioides* Penz. f. *stylosanthis* forma specialis *stylosanthis* Munaut f. sp. nov. found on most other *Stylosanthes* species.

RAPD analysis showed that the Australian type A isolate grouped with the Mexican isolates obtained from *S. humilis* and with all but one of the isolates obtained from *S. viscosa*. The Australian type B isolate clustered with all but one of the Mexican isolates found on *S. guianensis*. Within each of these main clusters, the Mexican isolates were more closely related to each other than to the Australian ones, suggesting distinct genetic evolution of *C. gloeosporioides* populations according to their geographical origin. Two African isolates differing from the typical type A or B isolates (Munaut et al. 1998) clustered with the two remaining Mexican isolates from *S. viscosa* and *S. guianensis.* These results confirm that the types A and B occur in nature on wild *Stylosanthes* species in Mexico and that they present similar host species as in cultivated areas worldwide.

A second phenetic analysis was performed by separating the isolates according to their *Stylosanthes* species of origin (*S. viscosa*, *S. humilis* and *S. guianensis*) (Munaut 1999). The clustering of 74 strains found on wild *S. viscosa* plants was primarily linked to their population of origin, but also to the date of collection. The phenogram obtained therefore represents a dynamic evolution in *C. gloeosporioides* structure. The 27 isolates from *S. humilis* could be separated into four different types, partially related to the state of origin (Michoacan and Nayarit). The isolates collected in four populations in Nayarit clustered in two separate groups, without clear link to their population of origin, morphological or pathogenic differences. This lack of correlation is often observed in the *Stylosanthes-C. gloeosporioides* interaction (Chakraborty et al. 1997; Kelemu et al. 1997). Furthermore, the Australian isolate type A, which is obtained from *S. viscosa*, was more similar to the isolates from Mexican *S. viscosa* than to those from *S. humilis*, suggesting a common genomic basis for isolates from *S. viscosa*. This means that, although all the latter species belong to *C. gloeosporioides* f. sp. *stylosanthis,* there is still a kind of subpopulation of isolates identifiable

within this forma specialis that are more related to one host species than to another.

The 18 isolates from *S. guianensis* could be grouped according to the geographical origins of their host (Michoacan, Nayarit, Veracruz). Although few strains had been analysed for this host, their diversity was greater than that found on strains proceeding from *S. viscosa* and *S. humilis*. Although highly polymorphic, strains from different acervuli transferred from the same plant are still more closely related to each other than isolates from different plants.

Among the 150 other *Colletotrichum* strains, 72 falcate-spored strains were isolated. ITS sequencing of representative strains allowed identification of only one *C. dematium* isolate, all the other strains being identified as *C. capsici*. This last species is known as a pathogen of several tropical crops, such as *Capsicum* spp.

Given the sequences available in the EMBL database at the time of analyses, the remaining 78 straight-spored *Colletotrichum* strains could not be assigned to any known species, although some similarities could be observed. At least four groups of strains should be investigated more accurately, and could represent new species.

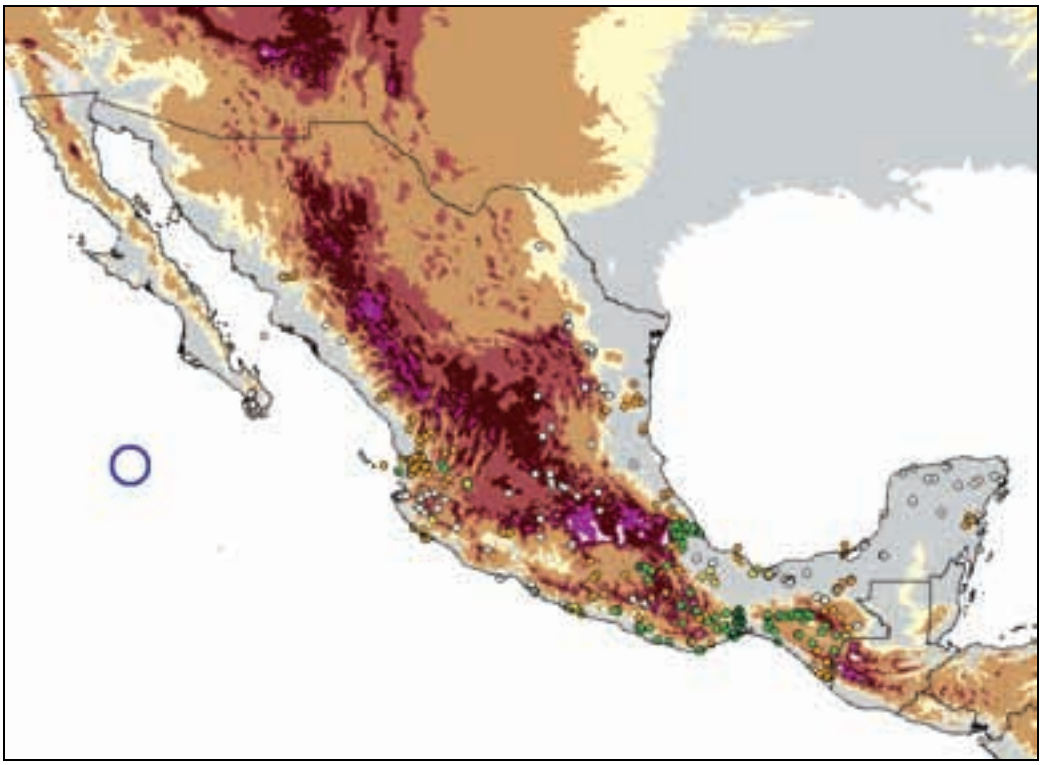
This extensive diversity study, performed on the five (or available) isolates obtained from the five acervuli on each plant, demonstrates the important diversity between isolates from different acervuli, plants and populations. Although pathogen diversity of the host species could be partially linked to the state where it was collected, isolates from plants collected in the same population were generally more similar to each other than to isolates from plants from different populations.

Even if significant genetic diversity and a corresponding high aggressivity of several *C. gloeosporioides* isolates was observed, *Stylosanthes* species are still widely distributed in Mexico and present a high level of species, morphological and molecular diversity (Vander Stappen et al. 1998, 1999a-b; Vander Stappen 1999). This suggests that there is a potential reservoir of resistance present in Mexican *Stylosanthes* populations, a potential that could be used by breeders for improvement of cultivated genotypes.

7. Conservation of *Stylosanthes* species in Mexico

The advances made during the research project provide a basis for the development of a strategy for the conservation and use of *Stylosanthes* genetic resources in Mexico and around the world. Crucial to any such effort is a solid taxonomic framework, which has been provided by the revision of the genus undertaken by UNAM. The distribution and ecology of the Mexican species is also better understood now. New species were found in the states of Jalisco, Puebla and Oaxaca. In terms of species-level diversity, the state of Oaxaca had the greatest number of *Stylosanthes*, with eight species, while Chiapas and Jalisco had six species each. In several areas of the states of Jalisco, Michoacán, Oaxaca, Chiapas and the Yucatán Peninsula, 'hybrid zones' of *Stylosanthes* were detected, indicating that, in narrow areas, genetically distinct *Stylosanthes* populations meet, cross-pollinate and produce hybrid progeny.

Map 6 shows the results of point-centred analysis of the distribution pattern of diversity. A circle with a radius of one degree (the size of the circle shown in blue on the map) was drawn around each collection point and a Shannon-Weaver diversity index calculated based on the presence of *Stylosanthes* species within the circle. Points in dark green show where species diversity is highest. An area in the south of the country has been highlighted as a hotspot of *Stylosanthes* species diversity, and thus probably a likely focus of any future *in situ* conservation work. Using GIS technology, it is now possible to assess the extent of *Stylosanthes* diversity within protected areas, and the risk it faces from different genetic erosion factors, such as urbanization or soil degradation.



Map 6. *Stylosanthes* species diversity in Mexico using a point-centred approach (see text for details). Dark green dots show highest diversity, white ones lowest, yellow and orange intermediate. The base map shows elevation.

Of course, the number of species is only one measure of diversity. Two areas may have equal numbers of species but the species may be more genetically similar among themselves in one area than in another. Based on the morphological, and especially the molecular marker data, we have a better idea of the genetic relationships among the species, and can augment the analysis of the geographical distribution of species diversity with this information. The molecular data also allows one to speculate about in which species, and the locations where, evolutionary processes are particularly active. We have found that the Mexican species from the *S. calcicola* lineage (or from phylogenetic clade 3) (cf. Section 3.3, Phylogenetic analysis of *Stylosanthes,* in this publication) seem to be speciating rapidly, and forming polyploids within the genus, in combination with the widespread species *S. viscosa* and *S. humilis*. These will make them particularly important targets for conservation, especially *in situ*.

Another important variable affecting conservation interventions is the pollinating system of the target species. The molecular data suggest that outcrossing is high in *S. guianensis* and *S. viscosa* and low in *S. humilis*. The balance between intrapopulation and interpopulation variation is likely to be somewhat different for outcrossers and inbreeders, with more population differentiation among the latter, which is reflected in the occurrence of regional types within each of these species.

Turning to *Colletotrichum,* which has been thoroughly studied at UCL, genetic diversity was found to be very high in Mexico compared with Africa and Australia, presenting a major danger for *Stylosanthes* cultivation. Anthracnose disease caused by *C. gloeosporioides* worldwide seems to represent only a small part of the infection potential demonstrated in the two formae speciales identified in Mexico. The results of the *Colletotrichum* work raise the question of the definition (and the existence) of the co-evolution phenomenon.

The results do not completely support the theory of co-evolution of host and pathogen. The huge diversity of the host plant *Stylosanthes* and its wide distribution suggests that the host plant is not threatened in its existence by the huge diversity, and phytopathological potential, of *C. gloeosporioides* and other *Colletotrichum* species—one could assume that there is a kind of balance between the host and its pathogen. If this can be considered as co-evolution, it is still an open question, as no analyses were undertaken of the resistance gene(s) in the host nor of the virulence gene(s) of the pathogen. Co-evolution of host and pathogen has long been assumed, particularly by the plant breeder community, and has been proven for host-pathogen combinations such as *Phaseolus* bean and the angular leaf spot fungus *Phaeoisariopsis griseola,* and for wheat and *Puccinia* rusts. This gene-for-gene interaction was the basis for resistance breeding, e.g. against cereal rusts.

Although significant genetic diversity was observed, as well as high aggressivity of several *C. gloeosporioides* isolates on wild *Stylosanthes* cuttings, the wide distribution and genetic diversity within the host suggests that a potential for resistance exists in Mexican material. The challenge for the future will be to limit the danger of movement of aggressive strains outside Mexico, while at the same time locating and transferring resistance to cultivated material.

8. References

Abang mm, Winter S, Green KR, Hoffman P, Mignouna HD, Wolf GA. 2002. Molecular identification of *Colletotrichum gloeosporioides* causing yam anthracnose in Nigeria. Plant Pathology 51: 63–71.

Alahakoon PW, Brown AE, Sreenivasaprasad S. 1994. Genetic characterization of *Colletotrichum gloeosporioides* isolates from mango. International Journal of Pest Management 40: 225–229.

Anderson LE. 1954. Hoyer's solution as a rapid permanent mounting medium for bryophytes. Bryologist 57: 242–244.

Anon[ymous]. 1937. Informaçoes sobre algumas plantas forrageiras. Ministerio da Agricultura, Rio de Janeiro, Brazil.

Atchison E. 1949. Studies in the Leguminosae. IV. Chromosome numbers and geographical relationships of miscellaneous Leguminosae. Journal of the Elisha Mitchell Science Society 65: 118–122.

Blake SF. 1920. Nine new plants of the genus *Stylosanthes*. Proceedings Biological Society Washington 33: 45–54.

Brandão FM. 1991. *Stylosanthes nunoi* Brandão, nova espécie do gênero *Stylosanthes* Sw. (Fabaceae), para o estado de Minas Gerais, Brasil. Daphne, Belo Horizonte 2: 5–7.

Brandão FM, Costa NMS. 1992. *Stylosanthes longicarpa* Brandão et Costa, uma nova contribuição para o gênero *Stylosanthes* Sw., no Brasil. Daphne, Belo Horizonte 2: 5–6.

Burkart A. 1939. Estudios sistemáticos sobre las Leguminosas – Hedisareas de la República Argentina y regiones adyacentes. Darwiniana 3: 232–261.

Burt RL. 1984. Natural variation in *Stylosanthes*. *In:* HM Stace and LA Edye (editors). The Biology and Agronomy of *Stylosanthes*. Academic Press, Sydney, Australia. pp. 103–123.

Burt RL, Edye LA, Williams WT, Gillard P, Grof B, Page M et al. 1974. Small-sward testing of *Stylosanthes* in northern Australia: preliminary considerations. Australian Journal of Agricultural Research 25: 559–575.

Burt RL, Edye LA, Williams WT, Grof B, Nicholson CHL. 1971. Numerical analysis of variation patterns in the genus *Stylosanthes* as an aid to plant introduction and assessment. Australian Journal of Agricultural Research 22: 737–757.

Cameron DF. 1967. Chromosome number and morphology of some introduced *Stylosanthes* species. Australian Journal of Agricultural Research 18: 375–379.

Cameron DF, Chakraborty S. 2004. Forage potential of *Stylosanthes* in different production systems. *In:* S Chakraborty (editor). High-yielding anthracnose-resistant *Stylosanthes* for agricultural systems. Australian Centre for International Agricultural Research (ACIAR), Canberra. pp. 27–38.

Chakraborty S. 2004. Anthracnose disease of *Stylosanthes*. *In:* S Chakraborty (editor). High-yielding anthracnose-resistant *Stylosanthes* for agricultural systems. Australian Centre for International Agricultural Research (ACIAR), Canberra. pp. 113–124.

Chakraborty S, Datta S. 2003. How will plant pathogens adapt to host plant resistance at elevated CO_2 under a changing climate? New Phytologist 159: 733–742.

Chakraborty S, Ratcliff D, McKay FJ. 1990. Anthracnose of *Stylosanthes scabra*: effect of leaf surface wetness on disease severity. Plant Disease 74: 379–384.

Chakraborty S, Perrott R, Charchar MJ, Fernandes CD, Kelemu S. 1997. Biodiversity, epidemiology and virulence of *Colletotrichum gloeosporioides*. II. Genetic and pathogenic diversity in isolates of *Colletotrichum* from eight species of *Stylosanthes*. Tropical Grasslands 31: 393–401.

Chakraborty S, Perrott R, Ellis N, Thomas MR. 1999. New aggressive *Colletotrichum gloeosporioides* strains on *Stylosanthes scabra* detected by virulence and DNA analysis. Plant Disease 83: 333–340.

Chakraborty S, Fernandes CD, Charchar MJ, Thomas MR. 2002. Pathogenic variation in *Colletotrichum gloeosporioides* infecting *Stylosanthes* spp. in a centre of diversity in Brazil. Phytopathology 92: 553–562.

Chakraborty S, Gosh R, Gosh M, Fernandes CD, Charchar MJ, Kelemu S. 2004. Weather-based prediction of anthracnose severity using artificial neural network models. Plant Pathology 53: 375–386.

Chang-Sun J, Hu-Se J, Xing-Rong M, Dong-Mei Z, Yi-Zheng Z. 2004. AFLP analysis of genetic variability among *Stylosanthes guianensis* accessions resistant and susceptible to the stylo anthracnose. Acta Botanica Sinica 2004: 480–488.

Cisar CR, Spiegel FW, TeBeest DO, Trout C. 1994. Evidence for mating between isolates of *Colletotrichum gloeosporioides* with different host specificities. Current Genetics 25: 330–335.

Cisar CR, TeBeest DO. 1999. Mating system of the filamentous ascomycete, *Glomerella cingulata.* Current Genetics 35: 127–133.

Conger AD, Fairchield LM. 1953. A quick-freeze method for making smear slides permanent. Stain Technology 28: 281–283.

Costa NMS, Ferreira MB. 1984. Some Brazilian species of *Stylosanthes*. *In:* HM Stace and LA Edye (editors). The Biology and Agronomy of *Stylosanthes*. Academic Press, Sydney, Australia. pp. 23–48.

Costa NMS, Van den Berg C. 2001. A new species of *Stylosanthes* Sw. (Leguminosae-Papilionoideae) from Guerrero, Mexico. Kew Bulletin 56: 239–241.

Costa NMS, Van den Berg C. 2003. A new species of *Stylosanthes* Sw. (Leguminosae-Papilionoideae) from Mato Grosso do Sul, Brazil. Kew Bulletin 58: 743–747.

Cox ML, Irwin JAG. 1988. Conidium and appressorium variation in Australian isolates of the *Colletotrichum gloeosporioides* group and closely related species. Australian Systematic Botany 1: 139–149.

Curtis MD, Cameron DF, Manners JM. 1995. Molecular evidence that diploid *Stylosanthes humilis* and diploid *Stylosanthes hamata* are progenitors of allotetraploid *Stylosanthes hamata* cv. Verano. Genome 38: 344–348.

Dale JL, Manners JM, Irwin JAG. 1988. *Colletotrichum gloeosporioides* isolates causing different anthracnose diseases on *Stylosanthes* in Australia carry distinct double-stranded RNAs. Transactions of the British Mycological Society 91: 671–676.

Davis RD. 1987. Seed-borne *Colletotrichum gloeosporioides* infection and fungicidal control in *Stylosanthes* spp. Seed Science and Technology 15: 785–791.

Davis RD, Irwin JAG, Cameron DF. 1984. Variation in virulence and pathogenic specialization of *Colletotrichum gloeosporioides* isolates from *Stylosanthes scabra* cvv. Fitzroy and Seca. Australian Journal of Agricultural Research 35: 653–662.

Davis RD, Irwin JAG, Cameron DF, Shepherd RK. 1987. Yield losses caused by *Colletotrichum gloeosporioides* in three species of *Stylosanthes*. Australian Journal of Experimental Agriculture 27: 67–72.

Davis RD, Boland RM, Howitt CJ. 1992. Colony descriptions, conidium morphology, and the effect of temperature on colony growth of *Colletotrichum gloeosporioides* isolated from *Stylosanthes* spp. growing in several countries. Mycological Research 96: 128–134.

Denoyes-Rothan B, Guérin G, Délye C, Smith B, Maymon M, Freeman S. 2003. Genetic diversity and pathogenic variability among isolates of *Colletotrichum* species from strawberry. Phytopathology 93: 219–228.

Edgerton CW. 1914. Plus and minus strains in the genus *Glomerella*. American Journal of Botany 1: 244–254.

Edye LA. 1997. Commercial development of *Stylosanthes* pastures in northern Australia. I. Cultivar development within *Stylosanthes* in Australia. Tropical Grasslands 31: 503–508.

Edye LA, Burt RL, Nicholson CHL, Williams RJ, Williams WT. 1974. Classification of the *Stylosanthes* collection 1928–1969. CSIRO Austral. Div. Trop. Agron. Tech. Pap. No. 15. 28 p.

Ellis MA, Ferguson JE, Grof B, Sinclair JB. 1976. Transmission of *Colletotrichum gloeosporioides* and the effect of sulphuric acid scarification on internally borne fungi in seeds of *Stylosanthes* spp. Plant Disease Reporter 60: 844–846.

Erdtman G. 1943. An Introduction to Pollen Analysis. The Ronald Press Co., New York, USA.

Ferreira MB, Costa NMS. 1979. O gênero *Stylosanthes* Sw. No Brasil. Empresa de Pesquisa Agropecuaria de Mina Gerais, EPAMIG, Belo Horizonte. 107 p.

Freeman S, Katan T, Shabi E. 1998. Characterization of *Colletotrichum* species responsible of anthracnose diseases of various fruits. Plant Disease 82: 596–605.

Freeman S, Minz D, Jurkevitch E, Maymon M, Shabi E. 2000. Molecular analyses of *Colletotrichum* species from almond and other fruits. Phytopathology 90: 608–614.

Freeman S, Minz D, Maymon M, Zveibil A. 2001. Genetic diversity within *Colletotrichum acutatum sensu* Simmonds. Phytopathology 91: 586–592.

Gama-López S. 2006. Estudio sistemático del género Stylosanthes (Fabaceae). MSc thesis. Universidad Nacional Autónoma de México, Mexico.

García VA. 1990. Técnicas y procedimientos de citogenética vegetal. Universidad Autónoma de Chapingo, Chapingo, México. 196 p.

Gillies ACM, Abbott RJ. 1996. Phylogenetic relationships in the genus *Stylosanthes* (Leguminosae) based upon chloroplast DNA variation. Plant Systematics and Evolution 200: 193–211.

Gillies ACM, Abbott RJ. 1998. Evaluation of random amplified polymorphic DNA for species identification and phylogenetic analysis in *Stylosanthes* (Fabaceae). Plant Systematics and Evolution 211: 201–216.

Glover BJ, Gillies ACM, Abbott RJ. 1994. Use of the polymerase chain reaction to investigate the delimitation of two agronomically important species of *Stylosanthes* (Aubl.) Sw. Botanical Journal of Scotland 47: 83–96.

Guodao L, Phaikaew C, Stur WW. 1997. Status of *Stylosanthes* development in other countries: II. *Stylosanthes* development and utilisation in China and south-east Asia. Tropical Grasslands 31: 460–467.

Hayden HL, Pegg KG, Aitken EA, Irwin JAG. 1994. Genetic relationships as assessed by molecular markers and cross infection among strains of *C. gloeosporioides*. Australian Journal of Botany 42: 9–18.

Herter WG. 1943. Plantae Uruguayense novae vel criticae. Revista Sudamericana de Botánica 7: 209–210.

Hodson A, Mills PR, Brown AE. 1993. Ribosomal and mitochondrial DNA polymorphisms in *Colletotrichum. gloeosporioides* isolated from tropical fruits. Mycological Research 97: 329–335.

Hsiang T, Goodwin PH. 2001. Ribosomal DNA sequence comparisons of *Colletotrichum graminicola* from turf grasses and other hosts. European Journal of Plant Pathology 107: 593–599.

Irwin JAG. 1989. Diseases of pasture legumes in Australia. *In:* GC Marten, AG Matches, RF Barnes, RW Broughman, RJ Clements and GW Sheath (editors). Persistence of forages legumes. American Society of Agronomy, Madison, Wisconsin, USA. pp. 399–418.

Irwin JAG, Cameron DF. 1978. Two diseases in *Stylosanthes* spp. caused by *Colletotrichum gloeosporioides* in Australia, and pathogenic specialization within one of the causal organisms. Australian Journal of Agricultural Research 29: 305–317.

Irwin JAG, Cameron DF, Lenné JM. 1984. Responses of *Stylosanthes* to anthracnose. *In:* HM Stace and LA Edye (editors). The Biology and Agronomy of *Stylosanthes*. Academic Press, Sydney, Australia. pp. 295–310.

Javaux, C. 1998. Identification et étude de la diversité de *Colletotrichum capsici* isolé sur *Stylosanthes* sp. au Mexique. MSc thesis. Université catholique de Louvain, Louvain-la-Neuve, Belgium.

Johnston PR. 2000. The importance of phylogeny in understanding host relationships within *Colletotrichum* sp. In: D Prusky, S Freeman and MB Dickman (editors). *Colletotrichum*: host specificity, pathology, and host-pathogen interaction. American Phytopathological Society Press, St. Paul, MN, USA. pp. 21–28.

Johnston PR, Jones D. 1997. Relationships among *Colletotrichum* isolates from fruit-rots assessed using rDNA sequences. Mycologia 89: 420–430.

Kazan K, Manners JM, Cameron DF. 1993a. Genetic relationships and variation in the *Stylosanthes guianensis* species complex assessed by random amplified polymorphic DNA. Genome 36: 43–49.

Kazan K, Manners JM, Cameron DF. 1993b. Genetic variation in agronomically important species of *Stylosanthes* determined using random amplified polymorphic DNA markers. Theoretical and Applied Genetics 85: 882–888.

Kelemu S, Badel JL, Moreno CX, Miles JW, Chakraborty S, Fernandes CD et al. 1997. Biodiversity, epidemiology and virulence of *Colletotrichum gloeosporioides*. I. Genetic and pathogenic diversity in *Colletotrichum gloeosporioides* isolates from *Stylosanthes guianensis.* Tropical Grasslands 31: 387–392.

Kelemu S, Skinner DZ, Badel JL, Moreno CX, Rodriguez MX, Fernandes CD et al. 1999. Genetic diversity in South American *Colletotrichum gloeosporioides* isolates from *Stylosanthes guianensis*, a tropical forage legume. European Journal of Plant Pathology 105: 261–272.

Kelemu S, Miles JW, Rao IM. 2004. Biotic and abiotic constraints to *Stylosanthes* production. *In:* S Chakraborty (editor). High-yielding anthracnose-resistant S*tylosanthes* for agricultural systems. Australian Centre for International Agricultural Research (ACIAR), Canberra. pp. 97–111.

Khudrathulla MD, Jagannath MS. 1998. Biocontrol of ixodid ticks by forage legume *Stylosanthes scabra* (Vogel). Indian Journal of Animal Science 68: 428–430.

Kirkbride JH, Kirkbride CG. 1987. Typification of *Stylosanthes* (Leguminosae) and its sections. Taxon 36: 455–458.

Klitgaard BB, Lavin M. 2005. Dalbergieae *sens. lat. In:* GP Lewis, B Schrire, B Mackinder and M Lock (editors). Legumes of the World. Royal Botanic Gardens, Kew, UK. pp. 307–335.

Koenig R, Gepts P. 1989. Allozyme diversity in wild *Phaseolus vulgaris*: further evidence for two major centres of genetic diversity. Theoretical and Applied Genetics 78: 809–817.

Lavin M, Pennington RT, Klitgaard BB, Sprent JI, de Lima HC, Gasson PE. 2001. The dalbergioid legumes (Fabaceae): delimitation of a pantropical monophyletic clade. American Journal of Botany 88: 503–533.

Lenné JM. 1986. Recent advances in the understanding of anthracnose of *Stylosanthes* in tropical America. *In:* Proceedings of the XV International Grasslands Congress, Kyoto, Japan.

Lenné JM. 1993. *Colletotrichum* diseases of legumes. *In:* JA Baily and MJ Jeger (editors). *Colletotrichum:* Biology, pathology and control. CAB International, Wallingford, UK. pp. 134–166.

Lenné JM, Brown AE. 1991. Factors influencing the germination of pathogenic and weakly pathogenic isolates of *Colletotrichum gloeosporioides* on leaf surfaces of *Stylosanthes guianensis*. Mycological Research 95: 227–232.

Lenné JM, Calderon MA. 1984. Disease and pest problems in *Stylosanthes*. *In:* HM Stace and LA Edye (editors). The Biology and Agronomy of *Stylosanthes*. Academic Press, Sydney, Australia. pp. 279–293.

Lenné JM, Sonoda RM. 1982a. The occurrence of *Colletotrichum* spp. and the pathogenicity of Florida and Australian isolates to *Stylosanthes* spp. Tropical Grasslands 13: 98–105.

Lenné JM, Sonoda RM. 1982b. Effect of anthracnose on yield of the tropical forage legume *Stylosanthes hamata*. Phytopathology 72: 207–209.

Lenné JM, Vargas A, Torres C. 1983. Descripción de las enfermedades de las principales leguminosas forrajeras tropicales. Audiotutorial and study guide. CIAT series 04 SP-03.03. Cali, Colombia.

Lewis WH. 1980. Polyploidy in angiosperms: Dicotyledons. *In:* WH Lewis (editor). Polyploidy: Biological relevance. Plenum, New York, USA. pp. 241–268.

Lewis GP, Schrire BD. 2003. Leguminosae or Fabaceae? *In:* BB Klitgaard and A Bruneau (editors). Advances in Legume Systematics, part 10: Higher Level Systematics. Royal Botanic Gardens, Kew, UK. pp. 1–3.

Lewis GP, Schrire B, Mackinder B, Lock M. 2005. Introduction. *In:* GP Lewis, B Schrire, B Mackinder and M Lock (editors). Legumes of the World. Royal Botanic Gardens, Kew. UK. pp.1–20.

Liu CJ. 1997. Geographical distribution of genetic variation in *Stylosanthes scabra* revealed by RAPD. Euphytica 98: 21–27.

Liu CJ, Musial JM. 1995. Restriction fragment length polymorphism detected by cDNA and genomic DNA clones in *Stylosanthes.* Theoretical and Applied Genetics 91: 1210–1213.

Liu CJ, Musial JM. 1996. Development and application of RFLP and STS in *Stylosanthes*. Online *in* Proceedings of the 8th Australian Agronomy Conference, Toowoomba, 1996 (http: //www.regional.org.au/au/asa/1996/)

Liu CJ, Musial JM. 1997. *Stylosanthes* sp. aff. *S. scabra*: a putative diploid progenitor of *Stylosanthes scabra* (Fabaceae). Plant Systematics and Evolution 208: 99–105.

Liu CJ, Musial JM. 2001. The application of chloroplast DNA clones in identifying maternal donors for polyploid species of *Stylosanthes.* Theoretical and Applied Genetics 102: 73–77.

Liu CJ, Musial JM, Thomas BD. 1999. Genetic relationships among *Stylosanthes* species revealed by

RFLP and STS analyses. Theoretical and Applied Genetics 99: 1179–1186.

Louis I, Cook RC. 1985. Conidial matrix and spore germination in some pathogens. Transactions of the British Mycological Society 84: 661–667.

Lourd M, Geiger JP, Goujon M. 1979. Les *Colletotrichum*, agents d'anthracnoses en Côte d'Ivoire. Caractéristiques morphologiques et culturales d'isolats de *Colletotrichum gloeosporioides* Penz. Annales de Phytopathologie 11: 483–495.

Lusembo P, Ebong C, Sabiiti EN, Saleem M, Tedla A, Ndikumana J. 1997. Dry and wet season performance of selected herbaceous legumes in Uganda. Tropical Grasslands 31: 556–560.

Ma ZY, Chandra A, Musial JM, Liu CJ. 2004. Molecular evidence that *Stylosanthes augustifolia* is the third putative diploid progenitor of the hexaploid *S. erecta* (Fabaceae). Plant Systematics and Evolution 248: 171–176.

Maass BL, 't Mannetje L. 2002. *Stylosanthes seabrana* (Leguminosae: Papilionoideae), a new species from Bahia, Brazil. Novon 12: 497–500.

Maass BL, Sawkins M. 2004. History, relationships and diversity among *Stylosanthes* species of commercial significance. *In:* S Chakraborty (editor). High-yielding anthracnose-resistant S*tylosanthes* for agricultural systems. Australian Centre for International Agricultural Research (ACIAR), Canberra. pp. 9–26.

Malter JP. 1984. Caractérisation de *Colletotrichum gloeosporioides* Penz. attaquant le *Stylosanthes* en Afrique [dissertation]. Université catholique de Louvain, Louvain-la-Neuve, Belgium.

't Mannetje L. 1969. *Rhizobium* affinities and phenetic relationships within the genus *Stylosanthes*. Australian Journal of Botany 17: 553–564.

't Mannetje L. 1977. A revision of varieties of *Stylosanthes guianensis* (Aubl.) Sw. Australian Journal of Botany 25: 347–362.

't Mannetje L. 1984. Considerations on the taxonomy of the genus *Stylosanthes*. *In:* HM Stace and LA Edye (editors). The Biology and Agronomy of *Stylosanthes*. Academic Press, Sydney, Australia. pp. 1–21.

Maraite H. 1993. L'anthracnose du *Stylosanthes*. Bulletin des Séances de l'Académie Royale des Sciences d'Outre-mer 39: 577–586.

Martinez-Culebras PV, Querol A, Suarez-Fernandez MB, Garcia-Lopez MD, Barrio E. 2003. Phylogenetic relationships among *Colletotrichum* pathogens of strawberry and design of PCR primers for their identification. Journal of Phytopathology 151: 135–1743.

Masel A, Braithwaite K, Irwin J, Manners J. 1990. Highly variable molecular karyotypes in the plant pathogen *Colletotrichum gloeosporioides*. Current Genetics 18: 81–86.

Melhem TS. 1966. Pollen grains of plants of the Cerrado XVII - Leguminosae - Lotoideae: Tribe Hedysarae. Ann. Acad. Brasileria de Ciencias 38: 485–495.

Miles JW. 1985. Evaluation of potential genetic marker traits and estimation of outcrossing rates in *Stylosanthes guianensis*. Australian Journal of Agricultural Research 36: 259–265.

Miles JW, Lascano CE. 1997. Status of *Stylosanthes* development in other countries. I. *Stylosanthes* development and utilisation in South America. Tropical Grasslands 31: 454–459.

Mohamed-Saleem MA, Adeoti AA. 1989. Screening stylo accessions for anthracnose tolerance. Preliminary report. ILCA Bulletin 34.

Mohlenbrock RH. 1958. A revision of the genus *Stylosanthes.* Annals of the Missouri Botanical Garden 44: 299–347.

Mohlenbrock RH. 1960. Recent studies in the Leguminous genus *Stylosanthes*. Rhodora 62: 340–343.

Mohlenbrock RH. 1963. Further considerations in *Stylosanthes* (Leguminosae). Rhodora 65: 245–258.

Mordue JEM. 1971. CMI Descriptions of pathogenic fungi and bacteria, No. 325 - *Glomerella cingulata*. Commonwealth Mycological Institute, Kew, UK.

Moriwaki J. 2003. Morphological and molecular characterization of *Colletotrichum boninense* sp. nov. from Japan. Mycoscience: 44: 47–53.

Munaut F. 1990. Etude histopathologique des relations compatibles et incompatibles entre *Colletotrichum gloeosporioides* Penz et *Stylosanthes guianensis* Swartz [dissertation]. Université catholique de Louvain, Louvain-la-Neuve, Belgium.

Munaut F. 1999. Diversity of *Colletotrichum* species from wild *Stylosanthes*, a challenge for anthracnose management of cultivated *Stylosanthes.* PhD thesis. Université catholique de Louvain, Louvain-la-Neuve, Belgium.

Munaut F, Maraite H. 1998. Conidium germination and appressorium penetration phases of *Colletotrichum gloeosporioides* on *Stylosanthes guianensis*. Journal of Phytopathology 146: 19–26.

Munaut F, Hamaide N, Maraite H. 1997. A standardized microdroplets inoculation technique for the analysis of the interactions between *Colletotrichum gloeosporioides* and *Stylosanthes guianensis.* Parasitica 53: 117-125.

Munaut F, Hamaide N, Vander Stappen J, Maraite H. 1998. Genetic relationships among isolates of *Colletotrichum gloeosporioides* from *Stylosanthes* spp. in Africa and Australia using RAPD and ribosomal DNA markers. Plant Pathology 47: 641-648.

Munaut F, Hamaide N, Maraite H. 2001. Molecular and morphological characterization of *Colletotrichum gloeosporioides* from native Mexican *Stylosanthes* species. Plant Pathology 50: 383–396.

Munaut F, Hamaide N, Maraite H. 2002. Genomic and pathogenic diversity in *Colletotrichum gloeosporioides* from wild native Mexican *Stylosanthes* spp., and taxonomic implication. Mycological Research 106: 579–593.

Ohashi H. 1971. A taxonomic study of the tribe Coronilleae (Leguminosae) with special reference to pollen morphology. Journal of the Faculty of Science, University of Tokyo, Section III 11: 25–92.

Partridge I, Middelton C, Shaw K. 1996. Stylos for better beef. Department of Primary Industries, State of Queensland, Information Series QI96010. Brisbane, Australia

Pengelly BC, Clem RL, Whitbread AM. 2004. The role of *Stylosanthes* spp. in mixed crop-livestock systems in Africa and Australia. *In:* S Chakraborty (editor). High-yielding anthracnose-resistant S*tylosanthes* for agricultural systems. Australian Centre for International Agricultural Research (ACIAR), Canberra. pp. 77–84.

Pilger RKF. 1901. *Stylosanthes guianensis* var. *pubescens*. *In:* A Engler (editor). Bot. Jahrb. Syst. 30. pp. 160.

Pire SML. 1974. Estudio palinológico de la tribu Hedysareae (Leguminosae). Bonplandia 3: 143–169.

Polhill RM, Raven PH, Stirton CH. 1981. Evolution and systematics of the Leguminosae. *In:* RM Polhill and PH Raven (editors). Advances in Legume Systematics, part 1. Royal Botanic Gardens, Kew, UK. pp. 1–26.

Ramesh CR, Gangaiah B. 2004. Yield losses caused by *Colletotrichum gloeosporioides* in three species of *Stylosanthes* in India. *In:* S Chakraborty (editor). High-yielding anthracnose-resistant S*tylosanthes* for agricultural systems. Australian Centre for International Agricultural Research (ACIAR), Canberra. pp. 197–201.

Ramesh CR, Mal B, Hazra CR, Sukanya DH, Ramamurthy V, Chakraborty S. 1997. Status of *Stylosanthes* development in other countries. III. *Stylosanthes* development and utilisation in India. Tropical Grasslands 31: 467–475.

Ramesh CR, Chakraborty S, Sukanya DH. 2004. Role of native *Stylosanthes fruticosa* in the diversity of *Colletotrichum gloeosporioides* in India. *In:* S Chakraborty (editor). High-yielding anthracnose-resistant S*tylosanthes* for agricultural systems. Australian Centre for International Agricultural Research (ACIAR), Canberra. pp. 179–186.

Rudd VE. 1981. Aeschynomeneae. *In:* RM Polhill and PH Raven (editors). Advances in Legume Systematics, part 1. Royal Botanic Gardens, Kew, UK. pp. 347–354.

Sawkins MC. 1999. Geographic and genetic studies of *Stylosanthes* Sw. species. PhD thesis. University of Birmingham, Birmingham, UK.

Sawkins MC, Maass BL, Pengelly BC, Newbury HJ, Ford-Lloyd BV, Maxted N et al. 2001. Geographical patterns of genetic variation in two species of *Stylosanthes* Sw. using amplified fragment length polymorphism. Molecular Ecology 10: 1947–1958.

Schultze-Kraft R, Reid R, Williams RJ, Coradin L. 1984. The existing *Stylosanthes collections*. *In:* HM Stace and LA Edye (editors). The Biology and Agronomy of *Stylosanthes*. Academic Press, Sydney, Australia. pp. 125–146.

Serrato-Valenti G, Cornara L, Fernando M, Modensi P. 1992. Structural and histochemical features of *Stylosanthes scabra* (Leguminosa; Papilionoideae) seed coat as related to water entry. Canadian Journal of Botany 71: 834–840.

Sherriff C, Whelan MJ, Arnold GM, Lafey J, Brygoo Y, Bailey JA. 1994. Ribosomal DNA sequence analysis reveals new species groupings in the genus *Colletotrichum*. Experimental Mycology 18: 121–38.

Sloane H. 1696. Catalogus plantarum quae in Jamaica sponte proveniunt. London.

Sonoda RM, Kretschmer AE, Brolman JB. 1974. *Colletotrichum* leaf spot and stem canker on *Stylosanthes* spp. in Florida. Tropical Agriculture 51: 75–79.

Sreenivasaprasad S, Mills PR, Meehan BM, Brown AE. 1996. Phylogeny and systematics of 18 *Colletotrichum* species based on ribosomal DNA spacer sequences. Genome 39: 499–512.

Stace HM. 1984. Genetic systems in *Stylosanthes*. *In:* HM Stace and LA Edye (editors). The Biology and Agronomy of *Stylosanthes*. Academic Press, Sydney, Australia. pp. 147–161.

Stace HM, Cameron DF. 1984. Cytogenetics and the evolution of *Stylosanthes*. *In:* HM Stace and LA Edye (editors). The Biology and Agronomy of *Stylosanthes*. Academic Press, Sydney, Australia. pp. 49–72.

Stace HM, Cameron DF. 1987. Cytogenetic review of taxa in *Stylosanthes hamata* sensu lato. Tropical Grasslands 21: 182–188.

Stebbins GL. 1971. Chromosomal Evolution in Higher Plants. Edward Arnold Ltd, UK.

Sutherst RW, Jones JJ, Schnitzerling HJ. 1982. Tropical legumes of the genus *Stylosanthes* immobilize and kill cattle ticks. Nature 295: 320–321.

Sutton BC. 1980. The Coelomycetes. Commonwealth Mycological Institute, Kew, U.K.

Sutton BC. 1992. The genus *Glomerella* and its anamorph *Colletotrichum*. *In:* JA Bailey and MJ Jeger (editors). Colletotrichum: Biology, Pathology and Control. CAB International, Wallingford, UK. pp. 1–26.

Swartz O. 1788. Nova genera & species plantarum. Prod. Veg. Ind. Occ. 108.

Taubert P. 1891. Monographie der Gattung *Stylosanthes*. Verh. Bot. Vereins Prov. Brandenburg 32: 1–34.

Thomas D. 1984. Global ventures in *Stylosanthes* I. South America. *In:* HM Stace and LA Edye (editors). The Biology and Agronomy of *Stylosanthes*. Academic Press, Sydney, Australia. pp. 451–466.

Trevorrow RM, Chakraborty S, Jansen PI. 1998. Anthracnose resistance in *Stylosanthes seabrana*. *In:* Agronomy – Growing a Greener Future. Proceedings of the 9th Australian Society of Agronomy Conference, Wagga Wagga, NSW, 20–23 July 1998. pp. 211–212.

Vander Stappen J. 1999. Development of molecular markers for screening genetic diversity in the tropical legumes genus *Stylosanthes.* PhD thesis. Katholieke Universiteit Leuven, Leuven, Belgium.

Vander Stappen J, Volckaert G. 1999. Molecular characterization and classification of *S. mexicana, S. macrocarpa, S. seabrana,* and *S. fruticosa* by DNA sequence analysis of two chloroplast regions. DNA Sequence 10: 199–202.

Vander Stappen J, Van Campenhout S, Gama Lopez S, Volckaert G. 1998. Sequencing of the internal transcribed spacer region ITS1 as a molecular tool detecting variation in the *Stylosanthes guianensis* species complex. Theoretical and Applied Genetics 96: 869–877.

Vander Stappen J, Weltjens I, Munaut F, Volckaert G. 1999a. Interspecific and progeny relationships in the genus *Stylosanthes* inferred from chloroplast DNA sequence variation. Comptes Rendus de l'Académie des Sciences Paris III. Life Sciences 322: 481-490.

Vander Stappen J, Weltjens I, Van Campenhout S, Volckaert G. 1999b. Genetic relationships among *Stylosanthes* species as revealed by sequence-tagged site markers. Theoretical and Applied Genetics 98: 1054–1062.

Vander Stappen J, Weltjens I, Volckaert G. 1999c. Microsatellite markers in *Stylosanthes guianensis.* Molecular Ecology 8: 513–517.

Vander Stappen J, Weltjens I, Gama-López S, Volckaert G. 2000. Genetic diversity in Mexican *Stylosanthes humilis* as revealed by AFLP, compared to the variability of *S. humilis* accessions from South American origin. Euphytica 113: 145–154.

Vander Stappen J, De Laet J, Gama-López S, Van Campenhout S, Volckaert G. 2002a. Phylogenetic analysis of *Stylosanthes* (Fabaceae) based on the internal transcribed spacer region (ITS) of nuclear ribosomal DNA. Plant Systematics and Evolution 234: 27–51.

Vander Stappen J, Gama-López S, Dávila P, Volckaert G. 2002b. Molecular evidence for the hybrid origin of a new endemic species of *Stylosanthes* Sw. (Fabaceae) from the Mexican Yucatán Peninsula. Botanical Journal of the Linnean Society 140: 1–13.

Vanni R. 1987. Recuentos cromosómicos en *Zornia*, *Stylosanthes* y *Desmodium* (Leguminosae). Bonplandia 6: 39–43.

Vanni R. 1995. *Stylosanthes recta* Vanni (Leguminosae-Aeschynomeneae), una nueva especie del Chaco Paraguayo. Candollea 50: 247–249.

Vieira MLC, de Aguiar-Perecin MLR, Martins PS. 1993. A cytotaxonomic study in twelve Brazilian taxa of *Stylosanthes* Sw., Leguminosae. Cytologia 58: 305–311.

Vieira MLC, Fungaro MHP, Jubier M, Lejeune B. 1997. Determination of taxonomic relationships among Brazilian taxa of *Stylosanthes* Sw., Leguminosae, using RAPD markers. Pesqui. Agropecu. Bras. 32: 305–310.

Vinijsanun T, Irwin JAG, Cameron DF. 1987. Host range of three strains of *Colletotrichum gloeosporioides* from tropical pastures legumes, and comparative histological studies of interactions between type B disease-producing strains and *Stylosanthes scabra* (non-host) and *S. guianensis* (host). Australian Journal of Botany 35: 665–677.

Vogel JRT. 1838. De Hedysareis Brasiliae: *Stylosanthes*. Linnaea 12: 63–71.

von Arx JA. 1957. Die Arten der Gattung *Colletotrichum* Cda. Phytopathologische Zeitschrift 29: 915–925.

Waller JM, Bridge PD. 2000. Recent advances in understanding Colletotrichum diseases of some tropical perennial crops. *In:* D Prusky, S Freeman and MB Dickman (editors). *Colletotrichum*: host specificity, pathology, and host-pathogen interaction. American Phytopathological Society Press, St. Paul, MN, USA. pp. 337–345.

Weeds PL, Chakraborty S, Fernandes CD, Charchar MJ, Ramesh CR, Kexian Y et al. 2003. Genetic diversity in *Colletotrichum gloeosporioides* from *Stylosanthes* spp. at centres of origin and utilization. Phytopathology 93: 176–185.

White TJ, Bruns T, Lee S, Taylor J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. *In:* MA Innis, DH Gelfand, JJ Sninsky and TJ White (editors). PCR protocols: a guide to methods and applications. Academic Press, San Diego, USA. pp. 315–322.

Whitkus R, de la Cruz M, Mota-Bravo L, Gomez-Pompa A. 1998. Genetic diversity and relationships of cacao (*Theobroma cacao* L.) in southern Mexico. Theoretical and Applied Genetics 96: 621–627.

Williams RJ, Burt RL, Strickland RW. 1976. Plant introduction. *In:* Some concepts and methods in tropical and subtropical pasture research. C.A.B. Bull. No. 51.

Williams RJ, Reid R, Schultze-Kraft R, Costa SNM, Thomas BD. 1984. Natural distribution of. *Stylosanthes*. *In:* HM Stace and LA Edye (editors). The Biology and Agronomy of *Stylosanthes*. Academic Press, Sydney, Australia. pp. 73–101.

Yang HA, Sweetingham MW. 1998. The taxonomy of *Colletotrichum* isolates associated with lupin anthracnose. Australian Journal of Agricultural Research 49: 1213–1223.

9. Project publications

Gama-López S, Vander Stappen J, Dávila Aranda P, Volckaert G. 2001. Systematic study of the genus *Stylosanthes* (Fabaceae). *In:* Legumes Down Under. Abstracts of the 4th International Legume Conference: Legumes Down Under, Canberra, Australia. pp. 26–27.

Maraite H. 1993. L'anthracnose du *Stylosanthes*. Bulletin des Séances de l'Académie Royale des Sciences d'Outre-Mer 39: 577–586.

Munaut F, Hamaide N, Maraite H. 1997. A standardized microdroplets inoculation technique for the analysis of the interactions between *Colletotrichum gloeosporioides* and *Stylosanthes guianensis.* Parasitica 53: 117-125.

Munaut F, Hamaide N, Maraite H. 2001. Molecular and morphological characterization of *Colletotrichum gloeosporioides* isolates from native Mexican *Stylosanthes* species. Plant Pathology 50: 383–396.

Munaut F, Hamaide N, Maraite H. 2002. Genomic and pathogenic diversity in Colletotrichum gloeosporioides from wild native Mexican Stylosanthes spp., and taxonomic implications. Mycological Research 106: 579–593.

Munaut F, Hamaide N, Vander Stappen J, Maraite H. 1998. Genetic relationships among isolates of *Colletotrichum gloeosporioides* from *Stylosanthes* spp. in Africa and Australia using RAPD and ribosomal DNA markers. Plant Pathology 47: 641-648.

Munaut F, Maraite H. 1998. Conidium germination and appressorium penetration phases of *Colletotrichum gloeosporioides* on *Stylosanthes guianensis*. Journal of Phytopathology 146: 19–26.

Munaut F, Petré B, Maraite H. 1994. Ultrastructure of appressoria penetration phases on *Stylosanthes guianensis* by *Colletotrichum gloeosporioides*. Parasitica 50: 95–102.

Vander Stappen J, De Laet J, Gama-López S, Van Campenhout S, Volckaert G. 2002. Phylogenetic analysis of *Stylosanthes* (Fabaceae) based on the internal transcribed spacer region (ITS) of nuclear ribosomal DNA. Plant Systematics and Evolution 234: 27–51.

Vander Stappen J, Gama-López S, Dávila P, Volckaert G. 2002. Molecular evidence for the hybrid origin of a new endemic species of *Stylosanthes* Sw. (Fabaceae) from the Mexican Yucatán Peninsula. Botanical Journal of the Linnean Society 140: 1–13.

Vander Stappen J, Marant S, Volckaert G. 2003. Molecular characterization and phylogenetic utility of the rDNA external transcribed spacer region in *Stylosanthes* (Fabaceae). Theoretical and Applied Genetics107: 291–298.

Vander Stappen J, Van Campenhout S, Gama-López S, Volckaert G. 1998. Sequencing of the internal transcribed spacer region ITS1 as a molecular tool detecting variation in the *Stylosanthes guianensis* species complex. Theoretical and Applied Genetics 96: 869–877.

Vander Stappen J, Volckaert G. 1999. Molecular characterization and classification of *S. mexicana, S. macrocarpa, S. seabrana,* and *S. fruticosa* by DNA sequence analysis of two chloroplast regions. DNA Sequence 10: 199–202.

Vander Stappen J, Weltjens I, Gama-López S, Volckaert G. 2000. Genetic diversity in Mexican *Stylosanthes humilis* as revealed by AFLP, compared to the variability of *S. humilis* accessions from South American origin. Euphytica 113: 145–154.

Vander Stappen J, Weltjens I, Munaut F, Volckaert G. 1999. Interspecific and progeny relationships in the genus *Stylosanthes* inferred from chloroplast DNA sequence variation. Comptes Rendus de l'Académie des Sciences Paris III. Life Sciences 322: 481-490.

Vander Stappen J, Weltjens I, Van Campenhout S, Volckaert G. 1999. Genetic relationships among *Stylosanthes* species as revealed by sequence-tagged site markers. Theoretical and Applied Genetics 98: 1054–1062.

Vander Stappen J, Weltjens I, Volckaert G. 1999. Microsatellite markers in *Stylosanthes guianensis.* Molecular Ecology 8: 513–517.

Project publications in progress

Gama-López S, Alvarado JL, Dávila P, Rendón B. Estudio palinológico del género Stylosanthes (Fabaceae) para México. (To be published in Acta Botánica).

Gama-López S, Dávila P. Stylosanthes quintana-roensis (Fabaceae: Papilionoideae), una nueva especie del Sureste de México. (In press in Novon).

Gama-López S, Dávila P. Una nueva categoría infraespecífica de Stylosanthes guianensis (Fabaceae: Papilionoideae), del Occidente de México. (In press in Brittonia).

Gama-López S, Dávila P. Una nueva especie de Stylosanthes tehuacanensis (Leguminosae: Papilionoideae), para el Valle de Tehuacan-Cuicatlán (Puebla-Oaxaca; México). (To be published in Brittonia).

Gama-López S, Dávila P. Stylosanthes pseudohumilis (Leguminosae: Papilionoideae), una nueva especie de México. (In press in Brittonia).

Gama-López S, Dávila P, Grether R. Fruit micro-morphology of Stylosanthes (Leguminosa, (Papilionoideae) and its systematic significance (To be published in Systematic Botany).

Gama-López S, Dávila P. Cytogenetic studies of Stylosanthes species from México. (To be published in Cytologia).

Gama-López S, Arias S, Dávila P. A comparative study of Stylosanthes in México (To be published in Systematic Botany)

PhD theses

Munaut F. 1999. Diversity of *Colletotrichum* species from wild *Stylosanthes*, a challenge for anthracnose management of cultivated *Stylosanthes.* PhD thesis. Université catholique de Louvain, Louvain-la-Neuve, Belgium.

Vander Stappen J. 1999. Development of molecular markers for screening genetic diversity in the tropical legumes genus *Stylosanthes.* PhD thesis. Katholieke Universiteit Leuven, Leuven, Belgium.

MSc theses

Gama-López S. 2006. Estudio sistemático del género Stylosanthes (Fabaceae). MSc thesis. Universidad Nacional Autónoma de México, Mexico.

Javaux C. 1998. Identification et étude de la diversité de *Colletotrichum capsici* isolé sur *Stylosanthes* au Mexique. MSc Thesis. Université catholique de Louvain, Louvain-la-Neuve, Belgium.