Cytogenetics, genetic diversity and phylogenetics of wild *Zea* species, with emphasis on *Zea nicaraguensis*.

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Cover: Teosinte ear, chromosomes, Teosinte seeds, phylogenetic tree, and DNA sequences of *Zea nicaraguensis*.
(photos: Carlos Loáisga and Pernilla Ellneskog-Staam)

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Abstract

Maize (*Zea mays* subsp. *mays*) is a member of the grass family *Poaceae* (*Gramineae*), together with many other important agricultural crops. The wild species commonly known as teosintes are the closest relatives of maize. Teosintes are wild grasses with a native distribution area from Mexico to Nicaragua and are an important genetic resource for maize improvement. Teosinte from Nicaragua (*Zea nicaraguensis*) is to a large extent an unutilized genetic resource and its properties in terms of desirable traits, such as water-lodging adaptation and disease resistance have to be phenotypically and genetically characterized.

The aims of this thesis were to verify the chromosome number and characterize the genetic diversity of Nicaraguan teosinte populations and to determine genetic structure, gene flow, morphological variation and phylogenetic relationships among Meso-American teosintes. The thesis includes results from cytological, genetic, morphological and phylogenetic studies. Cytogenetic studies were based on C-banding techniques. Microsatellite (SSR) markers and DNA sequences of cpDNA regions were used for genetic diversity and phylogenetic studies respectively, whereas morphological studies involved various quantitative morphological traits. The C-banding pattern revealed that *Z. nicaraguensis* is more similar to *Z. luxurians* than other teosintes and cultivated maize.

Among the Meso-American teosintes, *Z. diploperennis, Z. perennis* and *Z. nicaraguensis* showed the highest values in the number of rare and unique alleles and the data also indicated that the gene flow between *Z. nicaraguensis* and *Z. luxurians* has been more frequent than between other teosintes. The morphological characterization revealed that the traits of the number of lateral branch nodes bearing ears, the glume width, the number of tiller nodes bearing ears and the number of tillers per plant were the most important in discriminating between taxa and the principal component analysis grouped many traits around the *Z. nicaraguensis, Z. luxurians* and *Z. mays* subsp. *huehuetenangensis*. Finally, the variation among cpDNA sequences was not enough to give a definitive phylogenetic resolution among the five *Zea* species, but even so our results support the idea that *Z. nicaraguensis* could be treated as a separate species distinct from *Z. luxurians*.

Keywords: Zea nicaraguensis, chromosome, teosinte genetic diversity, microsatellites, gene flow, characterization, chloroplast, taxonomy, phylogenetic.

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Dedication In Memoriam

A mi asesor, Arnulf Merker

Compartimos pocos años, pero fueron suficientes para apreciarte por que fuistes un gran asesor. Siempre dispuesto a escuchar, a dar consejos, a resolver los problemas y ver más allá del presente.

Mi eterna gratitud, siempre te recordaré.

To my supervisor, Arnulf Merker

Although we only shared a few years, it was enough to appreciate you because you were a great supervisor. Always ready to listen, to give advice, to solve problems and to see beyond the present.

My endless gratitude, I will always remember you.

Carlos

Contents

List of Publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I Ellneskog-Staam P., Loáisiga C., & Merker A. (2007). Chromosome C-banding of the teosinte *Zea nicaraguensis* and comparison with other *Zea* species. *Hereditas* 144, 96-101.

II Loáisiga C., Kolodinska Brantestam A., Diaz O., Salomon B., & Merker A. (2010). Genetic diversity in seven populations of Nicaraguan teosinte (*Zea nicaraguensis* Iltis and Benz) as estimated by microsatellite variation. *Genetic Resources and Crop Evolution* 58: DOI:10.1007/s10722-010-9637-6.

III Loáisiga C., Rocha O., Kolodinska Brantestam A., Salomon B., & Merker A. (2011). Genetic diversity and gene flow in six accessions of Meso-American teosintes. *Genetic Resources and Crop Evolution* 59:10.1007/s10722-011-9671-2.

IV Loáisiga C., Benavides A., Rocha O., Kolodinska Brantestam A., Geleta M., Salomon B., & Merker A. Morphological similarities between teosintes (*Zea* subsp). From Meso-America (Submitted).

V Loáisiga C., Geleta M., Rocha O., Kolodinska Brantestam A., Merker A., & Salomon B. A molecular phylogeny of *Zea* based on cpDNA regions (Manuscript).

Papers I-III are reproduced with the permission of the publishers.

The contribution of Carlos Henry Loáisiga Caballero to the papers included in this thesis was as follows:

I Planted seed for all samples in greenhouse, collected roots directly from the pot and wrote manuscript together with co-authors.

II Planted seed for all samples in greenhouse, collected young leaves for DNA extraction, planned and carried out all laboratory work, analysed data and wrote the manuscript together with supervisors.

III Planted seed for all samples in greenhouse, collected young leaves for DNA extraction, planned and carried out all laboratory work, analysed data and wrote the manuscript together with supervisors.

IV Planned, set up and carried out the experimental field trial, scored and analysed data and wrote the manuscript together with supervisors.

V Planted seed for all samples in greenhouse, collected young leaves for DNA extraction, planned and carried out all laboratory work, analysed data and wrote the manuscript together with supervisors

1 Introduction

1.1 History of geneus *Zea*

Zea L. is a genus that comprises a group of annual and perennial grass species native to Mexico and Central America. Meso-America is a region between Nayarit state in Mexico and approximately the middle part of the Pacific coast of Nicaragua. This is recognized as a center of origin of world agriculture and the center of diversity for approximately 225 cultivated plant species (Vavilov, 1931; Hernandez, 1985; Ortega-Paczka, 2003; Engels et al., 2006). The most significant contribution of this center to the world is the maize crop, *Zea mays* L., Hernandez (1985).

The genus includes both the wild taxa commonly known as "teosintes" (Table 1) and the cultigen maize *Zea mays* subsp. *mays*. Since maize is an American crop, it was unknown to the Europeans until Columbus first encountered it on the island of Cuba. Knowledge about teosintes came to Europe much later, and it was only in 1832 that a German botanist, Schrader (1833), provided the annual form of teosinte with a scientific name, *Euchlaena mexicana* Schrader. In 1910, the American botanist, A. S. Hitchcock, discovered a perennial form of teosinte, which he named *Euchlaena perennis* Hitchcock (Hitchcock, 1922). These two grasses are so unlike maize in the appearance of their ears that their close relationship to maize was not recognized at first. But gradually, through the efforts of several botanists (Beadle, 1932; Collins, 1921; Harshberger, 1896), the close relationship between teosinte and maize was established. Reeves and Mangelsdorf (1942) gave formal recognition to this close relationship by transferring these species into the genus *Zea*, as *Z. mexicana* (Schrader) Kuntze and *Z. perennis* (Hitchcock) Reeves & Mangelsdorf.

The teosinte is generally considered to be the progenitor of maize. (*e.g.* Emerson & Beadle, 1932; Galinat, 1974; Darlington, 1963; Wilkes, 1972; Iltis, 1983a; Kato, 1984; McClintock et al., 1981; Doebley, 1984, 1990a, 2004).

Since the end of the 19th century, several hypotheses have been postulated to explain how maize originated. The most influential idea was the tripartite hypothesis of the 1930s (Mangelsdorf & Reeves, 1939), although it was discredited around the late 1970s, when the hypothesis of evolution of maize from teosinte had progressively won agreement among scientific communities (Beadle, 1972; Doebley, 1990a; Matsuoka et al., 2002a; Doolitle & Marbry, 2006).

There are two alternative hypothesis regarding the domestication and diversification of maize. The first hypothesis states that maize evolved at several domestication centers from different teosinte populations from 8000 years ago (i.e., multi-centric origin), whereas the second hypothesis states the unique event of maize domestication from teosinte populations of *Z. mays* subsp. *parviglumis* at Raza Balsas near Michoacan, Mexico (i.e., unicentric origin). These hypotheses are associated with the origin and diversification of maize and the different ways it adapted to the environment and to specific cultures (Kato et al., 2009). It is believed that maize evolution has resulted in between 220 to 300 races of maize on the American continents (Brown & Goodman, 1977; Vigouroux et al., 2008).

Teosintes grow in the valleys and mountains of the Meso-American region as wild plants in various habitats along streams and hillsides, and they can also be found as a weed in cultivated fields. For example, *Z. mays* subsp. *parviglumis* grows in the Balsas River drainage of southwest Mexico and for that reason it has been also called Blasas teosinte (Doebley, 2004), and *Z. nicaraguensis* grows around the forest peripheries where its stems and prop roots are covered by 10–40 cm of standing water, which is unique among teosintes (Iltis & Benz, 2000; Loaisiga et al., 2010).

Although maize and teosinte plants share a similar robust growth form, their female inflorescence or ears are strikingly different. The teosinte ear possesses only about 5 to 12 kernels, each sealed tightly in a stony casing. Collectively, the kernel and its stony casing are known as a fruitcase. At maturity, the teosinte ear disarticulates such that the individual fruitcases become the dispersal units. Protected within its casing, the teosinte kernel can survive the digestive tracts of birds and grazing mammals, enabling the seeds to be easily dispersed. Another factor to consider is that found in Nicaraguan teosinte, which grows in slow-moving water where the excess of flow water contributes to the dispersal of the seeds.

By comparison, the massive maize ear can bear 500 or more kernels, each of which is attached to the central axis of the ear or cob. The kernels are naked without adequate protection from predation and are easily digested by any animal that consumes them. Since the kernels are firmly attached to the cob and the ear does not disarticulate, a maize ear left on the plant will eventually fall to the ground with its full suite of kernels. When hundreds of maize kernels germinate so close to one another in the next season, the emerging plants are unable to develop normally (Sanchez et. al., 1998).

As stated above, the Nicaraguan teosinte is remarkable for its ability to grow in as much as 10-40 cm of standing or slow-moving water. For this reason, Iltis & Benz (2000) suggested that this species could provide maize

breeders with a potentially valuable source of germplasm that may lead to the development of maize capable of growing in water-logged soils.

Standley, (1950) reported a population of teosinte in Honduras in the mountains of the Department of Francisco Morazan, not far from Tegucigalpa, in the mountains of Copan, and even Nicaragua. However, this finding has not been verified by any other author. Loaisiga et al., (2010), could not find teosinte populations there and suggested that the population reported by Standley might be extinct. Nevertheless, this possible population was an important biological bridge between *Z. luxurians* and *Z. nicaraguensis*. Bearing in mind that many civilizations were established in this area (Meso-America), principally the Maya and Chorotega, it is likely that plant material was distributed by humans.

During the 19th century, the origin and evolution of Indian corn became a topic of great interest among botanists. The most perplexing fact that these scientists had to contend with was that maize, unlike the Middle Eastern cereals wheat, barley, and rye, apparently lacked an associated wild form that could be considered its ancestor. This situation provided ample room for speculation, but little hope for resolution of the ancestry of maize. However, the focus of the debate changed with the discovery of teosintes and the subsequent demonstration that some types of teosinte and maize formed fully fertile hybrids (Harshberger, 1896).

There are two hypotheses that have had their supporters over the last 50 years. There has also been a diversity of opinion within each of these two camps. Most researchers working in the field support the "teosinte theory" (Beadle 1972, 1980; Benz, 1987; deWet & Harlan, 1972; Doebley, 1983; Galinat, 1963; Iltis, 1983; Kato, 1984, McClintock et al., 1981). However, Mangelsdorf, (1974, 1986) never swayed in his belief that teosinte is not the ancestor of maize. Many analyses suggest that *Z. mays* subsp. *parviglumis* and maize share a more recent common ancestor than they do with the other teosintes, and the fact that teosinte is wild and maize is fully domesticated suggests that their common ancestor was also a teosinte (Doebley, 1990).

1.2 Systematics and cytogenetics of the genus *Zea*

Iltis & Doebley (1980) proposed a classification for *Zea*, which reflected its presumed evolutionary relationships. This system of classification was based on the morphological and ecological features of the taxa and includes four species, one of which has three subspecies. This was subsequently modified by Doebley (1990) and with the inclusion of the species describe by Iltis &

Benz (2000) the genus currently includes five species, one of which has four subspecies.

The basic chromosome number for the genus *Zea* is x=10. All species are diploid with the chromosome number 2n=2x=20 except for the tetraploid Z. *perennis* wich has 2n=4x=40. The general morphological characteristics and geographic distributions of all *Zea* taxa are summarized in Figure 1 and Table1.

1.2.1 *Zea diplopernis* Iltis, JF.Doebley and Guzman

Zea diploperennis (diploperennis teosinte) as its name signifies, is a diploid (2*n*= 2x=20) perennial species. It has a very narrow geographic distribution, being found only in a small region of the Sierra de Manantlán in the southwestern part of the state of Jalisco in Mexico. The number of tassel branches ranges from 2 to 10, normally more than *Z. perennis*, and the fruitcase is trapezoidal in shape.

1.2.2 *Zea perennis* (Hitchc.) Reeves and Mangelsd.

Zea perennis (perennial teosinte) is a tetraploid (2n=4x=40) perennial species and is the only polyploid species in the genus. It has a narrow geographic distribution on the northern slopes of Volcán de Colima in the state of Jalisco in Mexico. This species is quite similar to *Z. diploperennis*, but it can be distinguished from *Z. diploperennis* by being a more slender plant that lacks tuber-like short shoots and having a different ploidy level.

1.2.3 *Zea luxurians* (Durieu) RM.Bird

Zea luxurians (Guatemala or Florida teosinte) is a diploid (2n=2x=20) annual species native to southeastern Guatemala and Honduras. However, this species is now extinct in Honduras. It is also known from a single collection made in Oaxaca, Mexico in 1845. No one has been able to recollect it from this locality since that time, but there is now a recent report that it has been rediscovered (Cuevas, 2006), although this information remains to be verified. *Zea luxurians* differs from the two perennial species through its lack of rhizomes, but it shares other similarities with the two perennial species. The outer glumes of its male spikelets have numerous fine veins that distinguish it from all other *Zea* species. In this character it resembles the species of the sister genus, *Tripsacum*.

1.2.4 *Zea nicaraguensis* (Iltis and BF.Benz)

Zea nicaraguensis (Nicaraguan teosinte) is native to the Pacific coast of Nicaragua. It is a diploid (2n=2x=10) and grows at very low elevations compared to the altitudes for other teosinte species. This species has the ability to grow normally in almost 0.5 m of standing or slow-moving water. It is a maize-like, erect, candelabra-branched annual species. The outer glumes have two prominent keels that merge at the apex (9-10 mm long). Other characteristics are its transverse rugulose form, especially when young, the presence of stout basal tillers and prominent prop roots.

1.2.5 *Zea mays* L.

Zea mays is the species that comprises both cultivated maize and annual teosintes. Adhering to the biological species concept, Iltis & Doebley (1980) placed three annual teosintes in *Z. mays* emphasizing their close biological relationship to maize, since maize and these teosintes are easily cross-hybridized and their hybrids are fully fertile.

1.2.5.1 Zea *mays* subsp. *huehuetenangensis* (Iltis and JF.Doebley) JF.Doebley

Zea mays subsp. *huehuetenangensis* (Huehuetenango teosinte) is a variant of teosinte found in western Guatemala. This diploid (2n=2x=*10*) perennial subspecies is distinguished from the other subspecies of *Z. mays* by its longer life cycle, which takes 7-8 months from germination to seed maturation in its native habitat and its height is only comparable with that of *Zea nicaraguensis*. Its floral morphology is similar to *Z. mays* subsp. *parviglumis* (see below) and it is difficult to distinguish these two subspecies on the basis of their tassel and ear morphology alone.

1.2.5.2 *Zea mays* subsp. *mexicana* (Schrad.) Iltis

Zea mays subsp. *mexicana* (Mexican annual teosinte) is a diploid (2n=2x=10) perennial subspecies that has a rapid life cycle with only 4 months from germination to seed maturation, depending on the populations. It is found in central and northern Mexico, ranging from Puebla to the Nobogame Valley in Chihuahua. Its fruitcases and tassel spikelets are relatively large, and distinguish it from *Z. mays* subsp. *parviglumis* and *Z. mays* subsp.

huehuetenangensis. The populations in the Race Chalco Valley in Mexico commonly have dark red, hairy leaf sheaths. Populations throughout the Mesa Central (Race Central Plateau) typically have green to pale red, glabrous to sparsely hairy leaf sheaths. The northernmost population in the Nobogame Valley of Chihuahua (Race Nobogame) is the shortest and earliest flowering teosinte.

1.2.5.3 *Zea mays* subsp. *parviglumis* Iltis and JF.Doebley

Zea mays L. subsp. *parviglumis* (Mexican annual teosinte) is a diploid (2n=2x=10) perennial subspecies found in the valleys along the western escarpment of Mexico from Nayarit to Oaxaca. Populations are distributed along an east-west axis from Oaxaca to Jalisco. Populations in the center of this range in the Balsas River drainage are known as Race Balsas or Balsas teosinte. It takes about 6-7 months from seed germination to plant maturity. The plants typically have green to pale red, glabrous leaf sheaths. The epithet "parviglumis" means small glume, referring to the small tassel spikelets.

1.2.5.4 *Zea mays* subsp. *mays*

Zea mays subsp. *mays* (maize) differs significantly in plant and ear morphology from the three teosinte subspecies of *Z. mays*. Iltis & Doebley (1980) did not place maize and three teosintes together in *Z. mays* based on the absence of reproductive isolation alone. They also stressed the similarities in the structure of their tassels. For example, all of them have relatively few widely-spaced veins of the glumes of tassel spikelets compared to *Z. diploperennis*, *Z. perennis*, *Z. luxurians* and *Z. nicaraguensis*. Additionally, molecular characteristics tie maize and the three subspecies together.

The closet relatives to *Zea* are found in the sister genus *Tripsacum* (*Gramigrasses*). The *Tripsacum* species are rhizomatous perennials that range from eastern North America to South America (Cuttler & Anderson, 1941; Randolph, 1959; Randolph & Hernandez, 1947). The basic chromosome number is x=18 and most species are diploids (2n=2x=36) or tetrapolid (2n=2x=72) but triploids (2n=2x=54) and hexaploids (2n=2x=108) have also been reported (Berthaund et al., 2004; deWet & Harlam, 1976; Randolph, 1976).

Since *Tripsacum* is the most closely related genus to *Zea* it has held a prominent place in *Zea* research. Despite the fact that several botanists have studied it in varying depth over the past 60 years, *Tripsacum* remains a taxonomically difficult group due to the presence of different ploidy levels even within a species and due to the fact that some species are apparently facultative apomicts, which can give rise to minutely but consistently differentiated forms (deWet et al., 1981). Like maize and teosinte, *Tripsacum* is monoecious. *Tripsacum* has been suggested as a potential donor of genes for disease and insect resistance that may be used in improving maize.

Generally, the teosintes have larger chromosomes than those found in cultivated maize where they have an average length of 11.2 μm. Among the wild species, *Z. nicaraguensis* has the largest chromosomes, with an average length of 19.6 μm (Ellneskog-Staam et al., 2007). *Zea perennis* has the smallest average chromosome size and the smallest heterochromatic blocks (Tito et al., 1991).

Cytological studies have dealt mainly with aspects of meiotic behavior, and the information on the chromosome knob constitution of the perennial teosinte is limited as to the presence of knobs in different chromosomes. Information available about *Z. perennis* is that of Longley (1937) who showed pachytene chromosomes. Regarding *Z. diploperennis,* the first pachytene chromosome observations showed the presence of small and medium knobs (Kato, 1984; Pasupuleti & Galinat, 1982). Previous surveys of chromosome knob constitution in populations of the genus *Zea* have led to a new interpretation about the origin and evolution of cultivated maize and its relationship with the diverse teosintes (Kato, 1976, 1984; McClintock, 1978; McClintock et al., 1981).

Chromosome knobs are enlarged structures consisting of condensed heterochromatin on mitotic and meiotic chromosomes. They can be used for taxonomic studies in the genus *Zea* because they exhibit fixed numbers and positions on chromosomes within specific taxa, but vary between taxa (Kato, 1976). In most cases, the main chromosome knobs in maize are internal or subterminal. In contrast, the chromosomes of *Z. nicaraguensis, Z. luxurians, Z. diploperennis,* and *Z. perennis* (Section *Luxuriantes*) have many terminal chromosome knobs and lack internal knobs. The main cytological difference between the *Z. nicaraguensis* and *Z. luxurians* genomes is that chromosome 10 of *Z. nicaraguensis* is knobless (Ellneskog-Staam et al., 2007).

On the other hand, all of the subspecies of *Z. mays* (Section *Zea*), in general, have many internal knobs and few or no terminal knobs except *Z.*

mays subsp. *huehuetenangensis,* which has many terminal chromosome knobs (Wilkes, 1967). These chromosomal knobs contain thousands to millions of tandem 180 bp and 350 bp repetitive DNAs that have high frequencies of preferential segregation as a result of meiotic drive (Buckler et al., 1999).

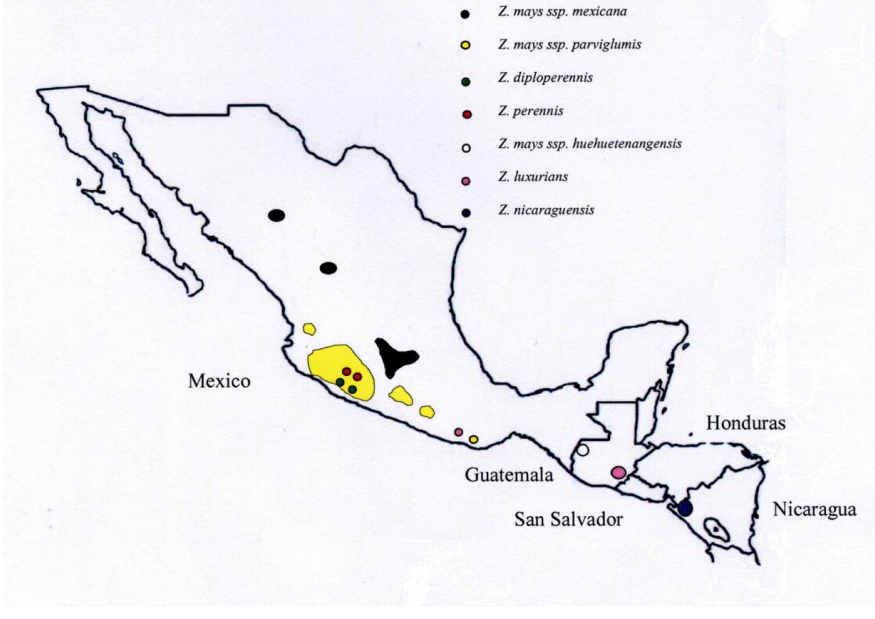

Figure 1. Distribution of teosinte in Mexico and Central America (Adapted from J. Doebley, 1990a).

Table 1. A summary of the principal characteristics of Meso-American teosintes.

Trait/taxa	Z. mays subsp mexicana	Z. mays subsp parviglumis	Z: masy subsp huehuetenangensis	Z. diploperennis	Z. perennis	Z. luxurians	Z. nicaraguensis
Habitat	Annual	Annual	Annual	Perennial	Perennial	Annual	Annual
Height	1.5-4.0 m	2.0-5.0 m	Up 5.0 m	2.0-2.5 m	1.5-2.0 m	3.0-4.0 m	Up to 6.0 m
Tassel branches	Slender, 10-20 branches	Slender, 20-100 branches	Slender 20 or more branches	Thicker, lax 2-10 branches	Thicker, erect 2-8 branches	Thicker, erect 4-20 branches	Slender, lax 27-38 branches
Male spikelet mm	Paired, longer pedicels 6-10	Paired, 4-7 mm long, small size pedicels 4-7	Paired longer pedicels 5-7	Paired, 10 mm shorter pedicels 1.5-3.5	Shorter pedicels, 1.5-3.8	Shorter pedi-cels 1.3-3.5	Paired, shorter pedicels1.5-3.5
Female spikelet	Slender and distichous	Slender and distichous	Slender and distichous	Slender and distichous	Slender and distichous	Slender and distichous	Slender and distichous
Flowering date	September to October	September to October	Late November to January	September to October	September to October	September to October	Mid. October to November
Fruitcases mg	Large, acute Tri angular 60-90	Small, blunt tri-angular 30-80	Small, triangular 30-60	Trapezoidal 68-75	Trapezoidal 70-83	Trapezoidal 76-99	Trapezoidal 76-99
Rhizomes mm	Absent	Absent	Absent	Slender, cord-like and tuber-like short internodes 2-6	Slender, cord-like with long inter-nodes 1-6	Absent	Absent
Ploidy	Diploid	Diploid	Diploid	Diploid	Tetraploid	Diploid	Diploid
Altitude	1700-2600	500-1800	900-1600	1400-2400	1500-2000	800-1100	10-20
Lat/long	20°05'-101°33	19°49'-104°11	15°40'-91°45	109°35'-104°12	19°38'-103°26	14°38'-89°38	12°53'-86°58

Adapted from Iltis et al., (1979); Iltis & Doebley (1980) and Loaisiga et al., (Unpublished).

1.3 Reproductive mechanisms and crossability among taxa

Hybridization between plant species is frequent and imports components of plant evolution and speciation (Rieseberg & Ellstrand 1993). More than 70 % of plant species may be descended from hybrids (Grant, 1981). Natural interspecific and in certain families, even intergeneric hybridization is not unusual and there are more than 1000 well studied examples (Grant, 1981; Arnold, 1997). Nonetheless, even if hybridization is common, it is not ubiquitous. The incidence of natural hybridization varies substantially among plant genera and families (Ellstrand et al., 1993).

The frequent occurrence of fertile hybrids increases the chances of introgression, i.e., the incorporation of alleles from one taxon into another (Richards, 1986). Studies employing allozymes and DNA based genetic markers have revealed many instances of natural introgression in plants. In many cases, morphological intermediacy and molecular confirmation of introgression go hand in hand. But, in other cases, one or few introgressed genetic markers may be found in otherwise morphologically typical individuals, even beyond the morphologically defined limits of a hybrid zone of contact (Rieseberg & Ellstrand, 1993; Runyeon et al., 1997).

Zea belongs to Tripsacinae, a subtribe of the Andropogoneae (Stebbins, 1950), which is a large tribe of tropical grasses that includes the other important economic plants sorghum (*Sorghum bicolor*) and sugar cane (*Sacharum officinalis*). Like most grasses, the Tripsacinae are wind-pollinated. Specifically, plants that are morphologically intermediate between maize and teosintes often occur spontaneously in and near Mexican maize fields when teosintes, particularly *Z. mays* subsp. *mexicana* are abundant (Wilkes, 1977). At present, the genetic data do not offer a clear view of the extent of hybridization and introgression that have occurred. Allozyme analysis of accessions of the teosintes *Z. luxurians, Z. diploperennis* and *Z. mays* subsp. *mexicana* revealed that alleles that are otherwise maize-specific occurred at extremely low frequencies, suggesting a very low level of introgression from maize into these teosinte taxa (Doebley, 1990).

However, cytogenetic analysis "offers no evidence of maize-teosinte introgression in either direction" (Kato, 1991). Allozyme comparisons of teosinte populations (involving *Z. diploperennis, Z. mays* subsp. *huehuetenangensis* and *Z. mays* subsp. *mexicana*) that are both allopatric and sympatric with maize cultivation showed no evidence of introgression from the crop into the wild taxa (Doebley, 1990a). Such comparisons are not available for *Z. mays* subsp. *parviglumis,* for which no taxon-specific

allozymes are available. To our knowledge, no one has genetically analyzed spontaneous, morphologically intermediate plants to test for hybridity in the same way as it was studied in rice. Such analysis would identify whether the maize-teosinte intermediates are true hybrids, introgressants, or crop mimics. Strong reproductive barriers exit between maize and *Z. perennis* and there is no evidence to suggest that natural hybridization occurs between these taxa (Doebley, 1990a).

The introgression of genes from a wild plant into a cultivated relative has been documented in several of our cultivated plants (barley, rice, sorghum and maize). There is hybridization between maize and teosinte in Mexico and adjacent regions of Guatemala (Huehuetenango), which is an example of reciprocal introgression, and varying amounts of maize germplasm is recognized in the six races of teosinte (Doebley, 1990a). Information regarding gene introgression between the recently discovered Nicaraguan teosinte and other *Zea* species is not available. Preliminary experiments with Meso-American teosintes in Nicaragua were carried out and showed good results: some hybrid seed is already available (Loaisiga, unpublished).

Doebley et al., (1987) examined isozymic evidence for introgression between maize and Mexican annual teosinte. Their data shows that maize and *Z. mays* subsp. *parviglumis* are nearly identical in isozyme allele constitution, and thus isozymes were not useful in the study of introgression between them. However, *Z. mays* subsp. *mexicana* possessed alleles distinct from those of maize at several loci. An examination of the distribution of these diagnostic alleles revealed that where maize is sympatric with *Z. mays* subsp. *mexicana*, some of these distinct alleles are also found in the cultigen, although at much lower frequencies than in *Z. mays* subsp. *mexicana* (Doebley, 1990a) and concluded that this distribution provides evidence for introgression from the wild form into the cultigen.

The rate of flow from other accessions and the final impact on the maize gene pool has been particularly difficult to measure (Fukunaga et al., 2005; Bellon et al., 2003; Pressoir & Berthaud 2004; Wilkes, 1969). In summary, the molecular data provides evidence for limited bidirectional gene flow between maize and teosinte. This evidence comes both from isozyme loci in the nuclear genome and distinct cpDNA genotypes of the chloroplast. Specifically, this data indicates low levels of gene flow from *Z. mays* subsp. *mexicana* into maize and from maize into *Z. diploperennis* and perhaps *Z. luxurians*.

Generally, there are five to nine seeds on spikes that shatter upon maturity for natural dispersal. A distinct difference between *Zea* and *Tripsacum* is that in *Zea* the male (staminate) flowers are produced in their own inflorescence (the tassel) that appears at the tips of the stalks and sometimes at the tips of ears, and the female (pistillate) flowers usually develop separately at the end of the lateral branches. On the other hand, in *Tripsacum* the male flowers are

usually only borne directly above the female flowers on the same spike. The flowering spikes with unisexual divisions are reproduced on numerous tall canes all originating at ground level from the basal rosette of leaves, in contrast to the teosinte plant habit of multiple branches that produces tiny ears in the leaf axils. The *Tripsacum* flowering shoot provides a genetic preview of the single stalk of domesticated maize (Iltis et al., 1979).

1.4 Conservation and utilization of *Zea* genetic resources

"Genetic resources", "biological resources", and "biodiversity" are terms that are frequently used in the literature of conservation. They are sometimes used ambiguously as having equivalent meanings but in fact they represent a progression (Allem, 2000). *In situ* gene banks are the largest repository of genetic resources and the least valued resource to many breeders because they value their holdings only if they have an identified use in a specific breeding program. In the same way, e*x situ* gene banks can also act as a large repository of conservation of genetic resources away from their natural habitat (Maxted et al., 1997). In both cases, the genetic resources in essence have the potential to add expanded genetic capabilities to the breeders' pool used to select better lines. They are valued because of what they might do, not because they have proven, established and defined value for the breeding process. The following quotation states the obvious: "If you want new genes, look in new places, but there is no guarantee" (Plucknett et al., 1987).

There is an obvious fundamental difference between these two concepts: *ex situ* conservation involves the sampling, transfer and storage of target taxa from the collecting area, whereas *in situ* conservation involves the designation, management and monitoring of target taxa where they are encountered. Another difference lies with the more dynamic nature of *in situ* conservation and the more static nature of *ex situ* conservation (Maxted et al., 1997). In both cases there are advantages and disadvantages, but the main reason for each system is as follows: *Ex situ* gene bank conservation is particularly appropriate for the conservation of crops and their wild relatives, while *in situ* gene bank conservation is especially appropriate for wild species and for landrace material on farms.

Biodiversity acknowledges the inter-working of many organisms to promote the proper function of the ecosystem. Biodiversity is inclusive of the genetic variance within interbreeding populations (genetic resources), and the trophic web relationships between taxa and the environmental amelioration of individual organisms. Genetic resources are ethereal; biological resources are the material expression of genetic resources, and

biodiversity is the effective source of multiple levels of biological resources. As the connections are seamless, they are often confused. Teosinte is a genetic resource for the maize genome, and wild teosinte is part of the biodiversity of the Meso-American region (Wilkes, 1997).

In the 1970s a discussion on the role of genetic resources and concerns about genetic erosion and the loss of landraces was initiated (Wilkes, 1997). Thirty years later, between two to five million *ex situ* accessions were available in over 100 gene banks worldwide. The total accessions world-wide in maize (*Zea*) amount to 277,000 and their relative (teosintes) totals more than 1000, concentrated in seven taxa (FAO 1996). It is of great importance to characterize these accessions so they will be used in crop specific breeding programs and also to account for the duplicate accessions. According to Wilkes (1997), the case for *in situ* conservation is not much more advanced than in 1970, with some exceptions, notably the wild relatives of maize in Mexico, Guatemala.

The discovery of the new diploid perennial taxon of teosinte, *Zea diploperennis* (Iltis et al., 1979) ultimately led to the establishment of the Sierra de Manantlán Biosphere Reserve. This sizable Biosphere Reserve encompasses a species and endemic taxon-rich, sub-tropical mountain forest, with two teosinte taxa and two sympatric landraces of maize, Tabloncillo and Reventador, found in the forest in small cultivated plots. The very persistence of the teosinte is dependent on the disturbed habitat and the fallow, abandoned maize fields (Benz, 1987). In fact, if the idea of maintaining "pristine" vegetation in the Biosphere Reserve were followed, the teosinte would probably become extinct (Benz, 1987).

Teosinte once formed a continuous or nearly continuous distribution along the Mexican–Nicaraguan escarpment between 20 and 1,500 meters, but is now found in nine geographically isolated clusters: six in Mexico, two in Guatemala and one in Nicaragua (Wilkes, 1967, 2004; Loáisiga et al., 2011). Some teosinte populations are small; the smallest occupies less than two km^2, whereas the largest covers hundreds of km^2. By best estimates, the current geographic distribution is less than half what it was in the 1960s. The extinction of remaining populations will accelerate as economics and land use intensify. Sanchez & Ruiz, (1996); Loáisiga et al., (unpublished) have identified deforestation, wildfire, urbanization (road building), and cattle as the major threats to teosinte.

The CIMMYT gene bank has committed to monitoring the extant teosinte populations of Mexico and Guatemala (Wilkes, 1985). This maize germplasm bank has the world's largest collection of maize landraces (50,895 accessions), along with the germplasm resources of the wild relatives (1,044 teosinte accessions) as living clones (*Tripsacum* spp.) or *ex situ* gene bank seed

(Biodiversity, 2007). The CIMMYT *in situ* monitoring effort was designed to watch for potential status changes of wild teosinte in natural habitats. This level of activity should not be confused with *in situ* conservation, which is a much more labor intensive and complicated endeavor. Today, maize is the first major crop for which any *in situ* monitoring of the status of its naturally occurring wild relatives has been undertaken on a long-term, regular basis, although Waines (1998) has proposed a similar activity for wheat in the Fertile Crescent (Damania, 1998). It was on the most recent monitoring trip 2004 that rapidly deteriorating conditions were discovered for the two populations in Guatemala and the Balsas teosinte 2005 found around Teloloapan, Guerrero (Mexico). Monitoring will do nothing to preserve teosinte populations, but it does give the opportunity to act before extinction takes place.

Teosinte in Guatemala (*Z. luxurians* and *Z. mays* subsp. *huehuetenangensis*) is near extinction and is expected to disappear from its natural habitats within the next decade if no immediate conservation efforts are undertaken (Wilkes, 2007). Three of the annual teosinte populations (*Z. nicaraguensis, Z. luxurians* and *Z. mays* subsp. *huehuetenangensis*) are considered "rare," occurring at single locations, while other are considered "vulnerable" (IUCN 1993).

Recently, Nicaragua received a grant under the Benefit-sharing Fund of the International Treaty on Genetic Resources for Food and Agriculture to start an *in situ* conservation program entitled; Rescue, conservation and sustainable management of teosinte (*Zea nicaraguensis* Iltis et Benz) in Apacunca Genetic Reserve (Loáisiga et. al., 2010). If no action is taken teosinte will go extinct. A farmer participatory plant breeding (FPPB) scheme will be initiated where one or two sites will continue to function as dynamic hybridization/evolutionary locations.

1.5 Maize domestication and breeding

Maize domestication has resulted in a highly modified inflorescence and plant architecture. Improvement after domestication has also resulted in striking changes in yield, plant habit, biochemical composition and other traits. Most domesticated plants have experienced a domestication bottleneck that reduces genetic diversity relative to their wild ancestor (Buckler et. al., 1999). This bottleneck affects all genes in the genome and modifies the distribution of genetic variation among loci. The magnitude and variance of the reduction in genetic diversity across loci provide insights into the demographic history of domestication (Innan and Kim, 2004).

Geneticists are now beginning to understand the problems of domestication bottleneck and the breeding history of a crop species (Hamblin et al., 2006; Hyten et al., 2006). One of the problems is the loss of useful genetic variation during the domestication process. Knowledge of the growth, physiology, and other various attributes of wild relatives may provide insights into key traits and allelic variants that are useful in modern agriculture (Tang et al., 2006). Several authors have proposed that changes in a small number of regulatory genes may be sufficient for the evolution of novel morphologies and that some teosinte genes played such a role during the morphological evolution of maize from its wild ancestors (Lukens et. al., 1999). Previous studies on the molecular level have demonstrated that domestication led to a loss of genetic diversity in maize.

In relation to nutritional components, maize is an important food and animal feed crop worldwide but low protein content in the kernel and the unbalanced amino acid composition, which lacks lysine and tryptophan, limit its nutritional value. Typical kernel composition values for the commodity yellow dent corn on a dry matter basis are 71.7% starch, 9.5% protein, 4.3% oil, 1.4% ash, and 2.6% sugar (Watson, 2003); 80% of the protein is stored in the endosperm, the nutritive tissue of the seed. It is reported that the grain protein content of wild relatives is significantly higher than those of cultivated crops (Fedack, 1984). In table 2, show some important amino acid composition in teosinte.

Several studies suggest that progenitors of modern maize contain a diversity of zein genes that is lacking in modern inbreeds (Swarup et al., 1995; Wilson & Larkins, 1984). For example, Swarup et al., (1995) found that exotic maize and wild members of the genus *Zea* exhibited higher levels of methionine-rich delta zeins than maize inbreeds, Wilkes, (1991), supposed that the high methionine trait was lost in the course of domestication. Whether loss of the high methionine trait was a result of artificial selection or random genetic drift is unclear.

The most outstanding progeny derived from hybrids of maize and *Zea perennis* had up to 50% higher protein content than commercial maize (Perini & Manoja, 1988). Perini et al., (1991) reported that a *Zea diploperennis* introgression population of maize had higher average protein levels than normal maize. Those studies show that alien germplasm introgression can produce beneficial effects on maize kernel quality. The *Z. mays* and *Z. mays* subsp. *parviglumis* subspecies differ substantially in plant, ear, and seed morphologies (Iltis, 2000). The most striking examples include differences in plant and inflorescence architecture conferred by the *teosinte branched1* locus (Doebley et al., 1995) and the hardened glume structure (fruitcase) surrounding the teosinte kernel conferred by *teosinte glume architecture1* (Dorweiler et al., 1993).

Teosinte populations with unique characteristics can serve as reservoirs of favorable alleles for the improvement of maize (Galinat, 1985a). Genetic studies of wild *Zea* species have already identified a series of potentially useful alleles for yield, water-logging tolerance, and perennialism. Using advanced backcross QTL (AB-QTL) analysis, valuable alleles for higher yield were identified and transferred from *Z. perennis* to maize (Harjes et al. 1999).

Duvik et al., (1999) made a critical assessment of traits that have contributed to improve the agronomic performance of maize since the introduction of the first hybrids, and several decades of breeding have brought noticeable changes in maize at the single plant level and crop level, for example; plant architecture (root, leaf and inflorescence, plant and ear height) lodging resistance (root lodging and stalk lodging), flowering time, maturity and growth.

Teosinte and landrace accessions may be sources of genetic variation for maize improvement, especially for genes that have limited or no variation remaining in modern inbred lines due to initial domestication events and plant breeding (Wright et al., 2005; Yamasaki et al., 2005). An example of the utility of wild species in genetic studies and crop improvement is using *Oryza rufipogon* grain yield in rice (Xiao et al., 1998). This is likely the case for the maize starch pathway, where three of six genes have experienced selection during domestication (Whitt et al., 2002).

Maize geneticists and breeders must work together to distinguish between selected and neutral genes, assay the allelic variation present in diverse inbreeds, landraces, and teosinte, and reintroduce these alleles into breeding programs in a manner that increases the efficiency of germplasm. Also is necessary to characterize the phenotypic diversity in agronomically relevant traits for the several gene pools, namely landraces and teosinte, in order to gain insight into which germplasm pools harbor valuable phenotypic variation (Wilkes, 2004). In the case of *Zea nicaraguensis*, it exhibits high ability to form adventitious roots at the soil surface during flooding (Bird, 2000) and tolerance to soil reducing or low redox potential conditions due to flooding (Mano & Omori 2008).

Table 2. Amino acid composition of defatted grain meals (g/100 g of protein). ⋆Paulis & Wal., (1977) and ⋆⋆ Loaisiga (unpublished).

Amino acid name	*Z. mays*[b]⋆	*Z. mays*[c]⋆	*Tripsacum dactyloides*⋆	*Z. diplo perennis*⋆⋆	*Z. nicaraguensis*⋆⋆
Lysine	2.8	2.3	1.4	1.8	1.7
Histidine[a]	2.9	2.5	2.5	2.4	2.5
Arginine[a]	4.7	4.1	3.2	3.4	3.7
Aspartate	6.8	7.1	5.6	6.9	7.5
Threonine[a]	3.7	3.3	3.3	3.7	4.2
Serine	5.1	5.1	5.0	5.9	6.7
Glutamic acid	20.5	21.2	22.9	25.0	28.1
Prolamine	8.7	7.9	8.6	9.7	11.3
Glycine	3.7	2.9	2.4	2.8	3.0
Alanine	8.1	8.8	10.1	9.8	10.8
Valine[a]	4.8	5.1	4.5	5.4	5.8
Cysteine	-	-	-	1.7	1.8
Methionine[a]	2.3	1.8	3.6	2.9	3.0
Isoleucine[a]	3.7	3.9	3.9	4.3	4.9
Leucine[a]	13.1	14.8	15.5	17.5	20.3
Tyrosine	4.7	5.3	5.0	4.5	4.1
Phenylalamine[a]	5.5	6.4	5.4	5.7	6.6
Ornitin	-	-	-	<0.1	<0.1
Sum	103.9	102.7		113.4	125.9
Ammoniac (NH_3)	2.8	3.1	3.3	3.3	3.7
Protein content	-	-	-	12.1	11.7

[a] Indispensable amino acid for non-ruminant animals and humans.
[b] Conventional maize.
[c] High protein content maize.
⋆ Extraction amino acids method in ⋆Paulis & Wal., (1977).
⋆⋆ Amino acids extraction data's: ISO-13903 method (Eurofins)

2. Objectives

The major objectives of this doctoral thesis were to study the cytogenetics, genetic diversity and phylogenetics teosinte species in the genus *Zea* with the ultimate goal of conserving and utilizing its genetic diversity. The following are the specific objectives of the study:

1. To verify the chromosome number of Nicaraguan teosinte (*Zea nicaraguensis*) and analyze the chromosome number in comparison with other teosintes in Meso-American region using C-banding technique.
2. To characterize the genetic diversity, population structure and gene flow of Nicaraguan teosinte populations and Meso-American teosintes accessions using microsatellite marker technique.
3. To estimate the level of genetic diversity and gene flow and determine the genetic structure of the Nicaraguan teosinte population using the microsatellite marker technique.
4. To characterize the extent and pattern of morphological variation in Meso-American teosintes using numerical taxonomic approaches.
5. To resolve the phylogenetic relationship among the Meso-American teosintes and maize, thereby determining the position of *Zea nicaraguensis* in the genus by using highly variable cpDNA.

3. Materials and methods

3.1 Plant material and DNA extraction

Plant material was provided by the CIMMYT Gene Bank in Mexico and the National Gene Bank (REGEN) of Nicaragua; seven accessions belonging to four species, one of which with two subspecies, were used in this study. The accessions are: two accessions of *Z. diploperennis*, two accessions of *Z. diploperennis, Z. perennis*, and one accession each of *Z. mays* ssp. *huehuetenangensis*, *Z. mays* ssp. *parviglumis*, *Z. luxurians* and *Z. nicaraguensis* and maize *Z. mays* subsp. *mays*. In addition, one accession of *Sorghum bicolor* (ZMB-7204) from the Zambian National Genetic Resource Centre, one accession of *Tripsacum dactyloides* var. *Meridonale (MIA 34536 PL)* and *(MIA 35920 PL)* from the *USA* were included. The geographic positions and altitudes of those accessions are given in Figure 1 and Table 3.

3.2 C-banding technique

Roots were taken directly from plants grown in the greenhouse for the chromosome preparation; the roots were excised when they were around 10 mm long and a standard cellulose-pectinase enzyme digestion method was used (Schwarzacher and Leitch, 1994). The chromosome C-banding was conducted followed the method described in Gill et al. (1991) and good cells were photographed with a Leica CCD digital camera. The details of this are given in paper I.

3.3 DNA amplification, electrophoresis, staining and data scoring

After optimizing the DNA amplification procedure, 21 maize microsatellite primer pairs were screened (www.maize.data.bank, www.maizegdb/ssr.php, www.teosinte.agron.missouri.edu/ssr), out of which 21 primers were used for the final analyses. SSR based DNA amplification; electrophoresis and staining of the amplified products were described in Paper II and III. In each analysis the set of bands were considered as codominant markers and were scored manually.

3.4 DNA sequencing of cpDNA regions

Eight fast-evolving cpDNA regions *psb*Z-*trn*G, *trn*Y-*psb*M, *trn*Y-*psb*M, *trn*Y-*trn*D, *rps*16-*trn*Q-1, *trn*V-*ndh*C, *ndh*F-*rp*132 and *pet*A-*psb*J, were amplified and sequenced using specific primers designed for this study. Details for amplifications, cleaning PCR products and sequencing are given in paper V.

3.5 Field trial

The experiments for morphological characterization of teosintes were conducted in Nicaragua in July 2008 at the Occidente experimental station in Chinandega, Nicaragua. The station is located at 80 masl altitudes, where precipitation ranges between 1800 and 2000 mm. The plot size for each accession was 2.88 m^2 with approximately 200 seeds per plot (6 rows, each 6 m long) and 15 individuals were used per accession. Each accession was characterized for 33 quantitative (Smith et al., 1981; Ramirez V. 1988; Sanchez et al., 1998; Doebley 1983). Those traits are given in Table 4 and Figure 2.

3.6 Data analysis

Data regarding chromosome length, chromosome arm ratio, length of large heterochromatic regions and position of regularly appearing thin heterochromatic bands was measured from each of three different individuals per species using the freeware computer program Micromeasure 3.3 (available at http: //www.colostate.edu/depts/biology/micromeasure). These measurements were used together with a visual examination of pictures from all ten individuals to construct karyograms. Microsatellites (SSR) and morphological data were analyzed using various statistical

programs. The details of genetic diversity parameters (Nei 1973, 1978 & Nei and Roychoudhury, 1973), genetic differentiation and multivariate analysis are described in paper II, III and IV.

NTSYSpc (Rohlf, 2000) was used for genetic distance calculation and dendogram construction. INFOSTAT Statistic Program Software V-1.6 (INFOSTAT, 2003) was used for Principal Component Analysis (PCA) and also for the further analysis of some of the outputs from the other software. POPGENE version 1.31 (Yeh & Boyle, 1997) was used for the analysis of percentage of polymorphic loci whereas Arlequin version 2 (Schneider, Roessli & Excoffier, 2000), was used for the analysis of molecular variance (AMOVA) and the INFOGEN program was used to make the Generalized Procrustes Analysis, GPA (Gower 1975).

DNA sequences data from eight cpDNA regions was edited using BIOEDIT version 7.0.5 (Hall, 2005) and SEQUENCES SCANNER version 1.0 (Apply Biosystems), DNA sequences were aligned using CLUSTAL X version 1.81 (Thomson et al., 1997), followed by manual adjustment, and the phylogenetic analysis of DNA sequences data was carried out using PAUP* 4.0 Beta 10 (Swofford, 2000).

Table 3. Number of individuals used for chromosome numbers, genetic diversity, gene flow, genetic relationship and cpDNA based phylogenetic analysis of eight accessions of Meso-American teosinte.

Taxa	C-banding	SSR[a]	Morphological[b]	sequencing[c]
Z. diploperennis-1	3	16	10	2
Z. diploperennis-2	--	17	--	2
Z. perennis-1	--	13	10	2
Z. perennis-2	--	14	10	2
Z. luxurians	3	14	10	2
Z. nicaraguensis	3	15	10	2
Z. mays subsp. mays	3	--		2
Z. mays subsp *huehuetenangensis*	--	15	10	2
Z. mays subsp. *parviglumis*	--	13	10	2
Tripsacum dactylides	--	--	--	2
Sorghum bicolor	--	--	--	1

[a]Twenty one microsatellite markers were used for each taxon.
[b]Thirty three morphological traits were evaluated for each taxon.
[c]Eight cpDNA regions (*psb*Z-*trn*G, *trn*Y-*psb*M, *trn*Y-*psb*M, *trn*Y-*trn*D, *rps*16-*trn*Q-1, *trn*V-*ndh*C, *ndh*F-*rp*132 and *pet*A-*psb*J) were used.

Table 4. Teosinte quantitative morphological characteristics evaluated in Meso-America.

X1a	Days to the beginning of flowering	X18b	Spike length (cm) *A-F
X2a	Days to complete flowering	X19b	Distance between branches in the tassel (cm) *D-E
X3a	Total number of leaves	X20b	Mean branch length in spike (cm) *A-D
X4a	Leaf length (cm)	X21b	Internode length in main branches in spike (cm) *B-C
X5a	Leaf width (cm)	X22b	Internode length in secondary branches in spike (cm) *G-H
X6a	Plant height (cm)	X23b	Spikelet width (mm) *A-B
X7a	First ear height (cm)	X24b	Spikelet length (mm) *C-D
X8a	Upper ear height (cm)	X25b	Pedicel length (mm) *D-E
X9a	Number of lateral branches in main stem	X26b	Glumes width (mm)
X10a	Number of tillers per plant	X27b	Number of veins per glumes
X11a	Number of nodes with ear in main stem	X28c	Ear length (cm)
X12a	Number of nodes with ear in the lateral branches	X29c	Number of grains in ear
X13a	Number of nodes with ear in the tillers	X30c	Hundred seeds weight (g)
X14a	Longitude on the 3rd uppermost last lateral branch	X31c	Grain length (mm)
X15a	Number of nodes on the 3rd uppermost one lateral branch	X32c	Grain width (mm)
X16a	Number of ears on the 3rd uppermost nodes of main stem	X33c	Number of seeds per plant
X17b	Number of branches in tassel		

*Details in Figure 2.

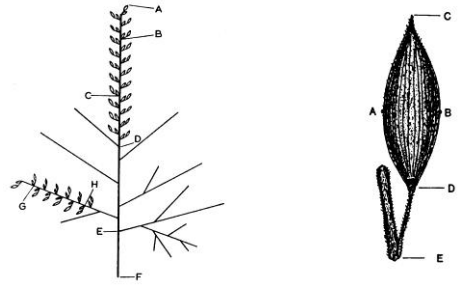

Figure 2. Diagrammatic representation of tassel and male pedicellate spikelet characteristics in Meso-American teosintes.

4. Summary of results and discussion

4.1 Chromosome comparisons in *Zea nicaraguensis* (paper I)

Chromosome knobs are enlarged structures consisting of condensed heterocromatin on mitotic and meiotic chromosomes. They can be used for taxonomy studies because they exhibit fixed numbers and positions on chromosomes within a specific taxon but vary between taxa (Kato, 1976). The chromosomal knobs contain thousands to millions of tandem 180 bp and 350 bp repetitive DNAs that have frequencies of preferential segregation as a result of meiotic drive (Wilkes, 1967).

The results in our study (paper I) revealed that *Z. luxurians* and *Z. nicaraguensis* have higher number of heterochromatic knobs than *Z. diploperennis* and *Z. mays* subsp. *mays*. Brown (1949) reported that the number of heterochromatic knobs in maize (*Z. mays* subsp. *mays*) decreases with increasing latitude in North America and this was confimed by Bennett (1976) who studied material of different latitudes from Mexico. Our results also showed this pattern because *Z. luxurians* and *Z. nicaraguensis* had a higher number of heterochromatic knobs than *Z. mays* subsp. *mays* and especially *Z. diploperennis,* which are from more northerly latitudes.

It is clear that *Z. nicaraguensis* and *Z. luxurians* share many features through all their chromosomes. For example, the location of heterochromatic areas is very similar, as shown by the C-banding pattern in chromosomes 1, 2, and 3 with long arm and short arm terminal bands and in chromosomes 4 to 9 with long arm and short arm terminal bands. The other two taxa, i.e., *Z. diploperennis* and *Z. mays* subsp. *mays*, also shared C-banding patterns that were markedly different from *Z. nicaraguensis* and *Z. luxurians.*

According to Kato (1976), chromosome 10 in *Z. luxurians* shows a larger arm ratio than those of other teosintes and maize and similar results were obtained by Longley (1973). Also in our study, *Z. luxurians* showed the larger arm ratio in chromosome 10 with 18.1 μm and *Z mays* the lowest with 9.3 μm. Tito et al. (1991) found *Z. perennis* to have the smallest average chromosome size and heterocromatic blocks. In contrast, the chromosome length in *Zea nicaraguensis* showed the highest average values for all chromosomes (19.6 μm) except for chromosome 10, only comparable with *Z. luxurians* in size.

In summary, the C-banding pattern in heterochromatic knobs was more pronounced in *Z. luxurians* and *Z. nicaraguensis* with 13 and 12, respectively, whereas *Z. diploperennis* and *Z. mays* subsp. *mays* showed only 9 knobs each.

The principal difference between *Z. luxurians* and *Z. nicaraguensis* was in chromosome 10 where only *Z. luxurians*. has heterochromatic knobs. This is a strong indication that *Z. luxurians* and *Z. nicaraguensis* are closely related species.

4.2 Genetic diversity among teosintes (paper II, III and IV)

From twenty two different maize primers used in this study, a subset of primers (11) was used in paper II and other subset primers (21) were used in paper III. All of them were polymorphic for all species, except three (*Bnlg* 1287, *Bnlg* 1305 and *Phi* 073) on accession *Z. diploperennis, Z. perennis* and *Z. luxurians* respectively. A total of 42 and 109 alleles were detected (paper II and III respectively).

The microsatellite repeats amplified were dinucleotides, trinucleotides or tetranucleotides. Trinucleotide microsatellites revealed the maximum number of alleles per loci 3.86, followed by dinucleotides with 3.51. Yao et al., (2008) found that the dinucleotide repeat motifs yielded more alleles per locus as compared to tri or tetranucleotides working on tea species.

In relation to the allele richness within different species through all nucleotides composition; *Z. huehuetenangenis* showed the lowest value (3.25) in observed alleles per locus, whereas *Z. diploperennis* showed the highest value (4.02). *Z. mays* subsp. *huehuetenangensis* showed the highest value in dinucleotides (4.0 observer number alleles) and *Z. diploperennis* the highest (5.0 and 4.02 observer number alleles) in trinucleotide and tetranucleotide respectively (Table 5).

Table 5. Observed and effective number of alleles among Meso-American teosinte accessions when classified into different microsatellite repeat motifs.

Nucleotide	Di nucleotide		Tri nucleotide		Tetra nucleotide		Average	
Taxa	na	ne	na	ne	na	ne	na	ne
Z. diploperennis	3.09	2.17	5.0	3.67	3.99	2.78	4.02	2.87
Z. perennis	3.54	2.38	3.66	2.99	3.87	2.9	3.69	2.75
Z. mays subsp. *huehuetenangensis*	4.0	2.84	2.5	1.96	3.25	2.29	3.25	2.36
Z. mays subsp. *parviglumis*	3.54	2.74	4.0	2.85	3.0	2.25	3.51	2.61
Z. luxurians	3.18	2.61	4.0	3.15	3.37	2.77	3.51	2.84
Z. nicaraguensis	3.72	2.59	4.0	2.82	3.25	2.32	3.65	2.57
Average	3.51	2.55	3.86	2.90	3.45	2.55	3.60	2.66

na: Observer number alleles ne: effective number alleles

When we studied all teosinte populations from Nicaragua, we found 19 rare alleles and 4 unique alleles. This genetic richness is more vulnerable to genetic erosion because all those alleles are present in low frequencies, even within each population. The AMOVA is congruent with these results because more than 80% of the genetic variation is within the populations (Table 5, paper II and Table 2, paper III). For this reason it is important to know the precise genetic structure of those populations, since genetic erosion is a dynamic process.

The hundreds of well studied cases of natural hybridization and introgression involving wild plants suggest that most domesticated plants will hybridize naturally with their cross-compatible wild relatives when they come into contact. A growing number of both experimental and descriptive studies, using genetically based markers, have demonstrated that domesticated alleles can and do enter and persist in natural populations (Ellstrand et al., 1999).

Due to the fragmentation of *Z. nicaraguensis* populations (the biggest area covers less than two hectares) and the fact that there are only a few individuals per population (5000 maximum plants in the bigger population), those populations are in difficulties, because these populations are a completely endemic species, as affirmed by Iltis et al., 2000; Bird, 1978; 2000; Benavides, 2003. In spite of this, the populations have a relative stable genetic structure or relative equilibrium, with 1.08 on average (Table 3, paper II).

Warburton et al., (2008) explain that normally the genetic diversity of maize landraces and teosintes is known to be partitioned mainly within populations and much less between populations; these indicate that each specific population conserves an intrinsic genetic variation. This is true if we observe the Figure 2 in paper II: all populations appear to be spread across a determinate space among quadrants, for example, population E is on average more concentrated in quadrant IV while population D is only found in quadrants I and III. This is supported by the AMOVA analysis, where genetic variation is high within populations but not between populations.

If we compare the average genetic diversity of *Z. nicaraguensis* (0.563), there is some variation. Matsuoka et. al., (2002) working with other teosintes found 0.50. Additionally, a total of 19 and 4 rare and unique alleles were found respectively (paper II). The genetic diversity in six Meso-American teosintes showed that the accessions *Z. diploperennis, Z. perennis* and *Z. nicaraguensis* had the highest values in terms of rare and unique alleles (11, 11 and 9 respectively); in contrast, *Z. luxurians, Z. mays* subsp. *parviglumis* and *Z. mays* subsp. *huehuetenangensis* showed the lowest values, less than 3 in both cases (paper III). These accessions might be more affected

by the genetic erosion than the others: since there are many alleles in low frequencies, if some of them disappear, they will disappear completely.

The teosintes are currently suffering a genetic bottleneck due to the fragmentation of populations and habitat loss (Warburton et al., 2008). Teosinte populations become fragmented and frequently become extinct due to overgrazing and other factors (Wilkes, 2006). Additionally, Van Heerwaarden et al., (2010), explain that in order to properly classify the diversity within populations, many individuals from each population should be sampled. Most studies of maize and teosinte have been done sampling few or most often only one individual per population. For all taxa there are strategic plans to preserve the original population, especially in Mexico and now Nicaragua with an *in situ* conservation program supported by IPGR-FAO.

In relation to genetic diversity, the accessions *Z. luxurians* and *Z. perennis* showed the highest values with 0.571 and 0.560 respectively, *Z. nicaraguensis* showed an intermediate rank and *Z. mays* subsp. *huehuetenangensis* and *Z. diploperennis* the lowest values (Table A3 in online resources). Doebley et al., (1984) obtained the same results with *Z. mays* subsp. *huehuetenangensis* and this is reasonable because this taxon showed a narrow geographical distribution and is endemic. Sanchez et al., (2000) and Matsuoka (2002) reported some variation in their results with the same taxa, using 46 microsatellite markers.

There are no correlations found between dendograms based on molecular and morphological data (Figure 4, paper III and Figure 4, paper IV). This can be partly explained by the fact that morphological data is affected by environmental conditions. The same problems have been experienced by other authors (Doebley & Iltis, 1980 and Smith & Lester 1980).

Previous papers (Wilkes, 1967; Kato, 1976; Senadhira, 1976; Orozco, 1979; Timothy et al., 1979; Iltis & Doebley, 1980; Mastenbroek et al., 1981; Smith et al., 1981, 1982) have shown that variation patterns and relationships within teosintes are complex, especially in *Z. mays* subsp. *mexicana*. Disagreements surrounding the taxonomy of teosintes and maize are due to the complex variation patterns within these taxa. Difficulties arise from various interpretations of similar data, preferential weighting of data, and discrepancies in variation patterns obtained from different data sets (Bird, 1982).

4.3 Genetic relationship and gene flow between teosintes (paper III and IV)

Gene flow from other teosinte subspecies and species certainly has occurred, since fertile hybrids can form between maize and several teosinte accessions,

and many teosintes grow in the same area (and even the same field) as maize in Mexico (Warburton et al., 2011).

Until now, there are only two reports about introgression involving *Z. nicarguensis* with other species. In the first of them, Warburton et al., (2011) found that maize individuals received nearly 10% of genomic diversity from the taxa *Z. luxurians, Z. nicaraguensis* and *Z. mays* subsp. *huehuetenangensis.* Secondly, Ross-Ibarra et al., (2009) reported that there are gene flows between *Z. luxurians* and *Z. nicaraguensis* and one or more subspecies of *Z. mays* at approximately 5% in six different loci.

Our molecular analysis found that the relationship between *Z. nicaraguensis* and *Z. luxurians* is greater than between *Z. nicaraguensis* and *Z. mays* subsp. *huehuetenangensis*. For example in the first pair, the genetic differentiation was F_{st} = 0.095; its value being lower than that of the second pair with F_{st} = 0.1304. In relation to gene flow, the first pair obtained N_m = 2.38 and the second revealed N_m = 1.667. When we compared the other combination pair (*Z nicaraguensis* and Z. *mays* subsp. *huehuetenangensis*), the genetic differentiation and gene flow were low (F_{st} = 0.1333 and N_m =1.629). Both indices showed more affinities or introgression between *Z. nicaraguensis* and *Z. luxurians* than between *Z. nicaraguensis* and *Z. mays* subsp. *huehuetenangensis*.

In other words, possibly gene flow was more frequent between *Z. nicaraguensis* and *Z. luxurians*, despite the smaller geographical distance between *Z. luxurians* and *Z. mays* subsp. *huehuetenangensis* (Figure 1). Those results are supported with cytological (Figure 1, paper I), molecular (Figure 4, paper III), morphological (Figure 4, paper IV) and sequences data as well (Figure 2). In all those figures, *Z. nicaraguensis* and *Z. luxurians* appear relatively close.

Some authors have argued that such introgression is a major force shaping the morphology and genetics of the various teosintes (Collins, 1921; Mangelsdorf, 1947; Wilkes, 1977; Bird, 1978). Other authors have contended that introgression is only a minor, unimportant factor, contributing little to variation among the teosintes (Kato, 1976; Doebley, 1984b). Most of the teosinte populations are spatially isolated by broken topography and high mountains and consequently the different populations may have acquired certain traits on morphological, ecological, chromosomal and genetic distinctness (Wilkes, 1977).

In the present study, a Generalized Procrustes Analysis (GPA) was used to compare the morphological and molecular data in clustering the Meso-American teosintes and to generate a consensus clustering pattern of the species. On average, 38% and 23.6% of the total variation was explained by the first and second axis respectively. Thus, the two axes explained 61.6% of

the total variation when values for the molecular and morphological data were averaged. However, when the two data sets were standardized and combined, the two axes explained 95.8% of the total variation (data not shown).

The accessions *Z. perennis* and *Z. diploperennis* showed a closer relationship between all accessions when both type of markers were compared, but they are even closer when the comparison is only with molecular markers. The case of *Z. nicaraguensis* and *Z. luxurians* showed more similarities using only morphological markers than the molecular markers. When using morphological data, for example, there is a clear difference between the group *Z. huehuetenangensis, Z. nicaraguenis* and *Z. luxurians* (taller plant and more vigorous) compared to the other group formed by *Z. pernnis, Z. diploperennis* and *Z. parviglumis* (short plants and small leaves).

Finally, *Z. mays* subsp. *huehuetanagensis* and *Z. parviglumis* were found to be very distant from each other based on either of the data sets, as shown in their respective independent analyses. Using consensus lines (black solid line), the perennial accessions showed a closer relationship to *Z. parviglumis* than to *Z. luxurians*, Z. *nicaraguensis* and *Z. mays* subsp. *huehuetenangensis* (Figure 2).

There are some differences between our results and Doebley et al., (1984) using molecular data. In their study, *Z. luxurians* appears between *Z. perennis* and *Z. mays* subsp. *huehuetenangensis,* but in the present study *Z luxurians* and *Z. nicaraguensis* are found in the same cluster. These results are congruent with Sanchez et al. (1998) and Matsuoka et al., 2002. These results are also supported in Figure 2, Paper II and Figure 4, Paper III, where *Z. luxurians* and *Z. nicaraguensis* are found in the same clade. Iltis et al., (1979), based on morphological data, and Timothy et al., (1982), in a cytoplasm genome study, placed those accessions in different positions.

The comparative morphological studies of male reproductive characters by Doebley & Iltis (1980) present the strongest arguments for considering that *Z. perennis* and *Z. diploperennis* have a closer relationship with *Z. luxurians* than with the annual Mexican teosintes. Comparative analyses of cytoplasmic DNA (Timothy et al., 1979; Sederoff et al., 1981) were unable to show a closer relationship of either *Z. perennis* or *Z. luxurians* with annual Mexican teosintes.

When we compared the morphological analysis between accessions, we found different arrangements among them. *Z. nicaraguensis* and *Z. luxurians* keep the same relationships, but the *perennial* teosintes appears in a different cluster. This discrepancy may be due to the Nicaraguan growing conditions.

Is important to consider that *Z. perennis* is a tetraploid, and thus not directly comparable to the diploids. However, it maintains substantial diversity, as can be seen from the spread of its populations on Figure 4,

paper III. Also, the average number of alleles in *Z. perennis* was 3.45, compared to 3.82 for *Z. diploperennis*; the lowest was *Z. luxurians* with 3.38 while *Z. nicaraguensis* had 3.62. Finally, the genetic identity that was most similar was between *Z. nicaraguensis* and *Z. luxurians*.

Those results highlight the high genetic diversity in teosintes but in particular in the *Z. nicaraguensis*, which one present more allele's richness than other teosintes, and how the gene flow factors might have had implications on the propagation and evolution in teosintes and the similarities/dissimilarities with *Z. luxurians*.

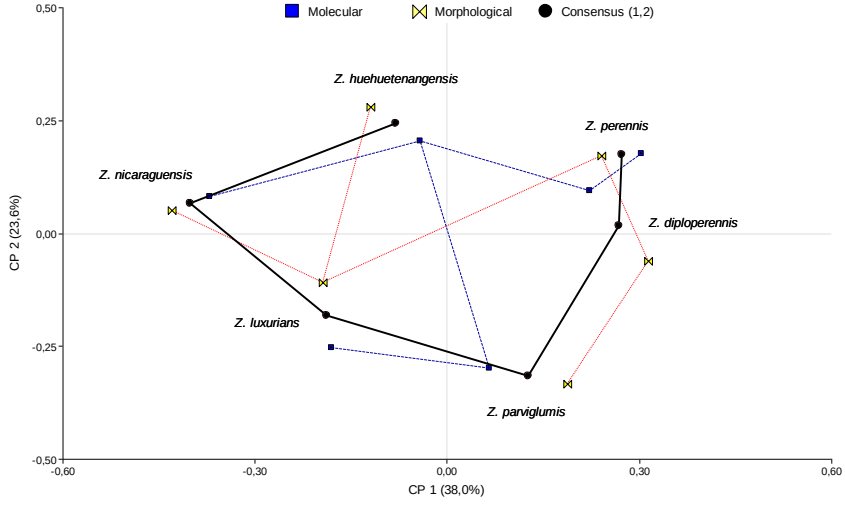

Figure 3. Generalized Procrustes Analysis (GPA) in Meso-American teosintes using morphological and molecular marker data.

4.4 *Zea nicaraguensis* and its position in the genus (paper V)

The results presented in paper V revealed two strongly supported clades (Figure 2). The species *Z. perennis, Z. diploperennis, Z. luxurians* and *Z. nicaraguensis* formed one clade, which is identical to section *Luxuriantes,* and the second clade was formed by *Z. mays*, i.e., *Z. mays* subsp. *huehuetenangensis, Z. mays* subsp. *mays* and *Z. mays* subsp. *parvilgimis*, which corresponds to section *Mays.* Thus, our data fully supports the current classification as described above.

However, our data does not resolve the relationships within section *Luxuriantes* since all four species appear as a basic unresolved polytopy. The data provided by Doebley et al. (1987), Buckler et al. (1996) and Fukunaga et al. (2005) suggest that the taxa in section *Luxuriantes* form two sister species pairs, i.e., *Z. perennis* and *Z. diploperennis* on the one hand and *Z.*

luxurians and *Z. nicaraguensis* on the other. Our cpDNA data can neither support nor refute any of the suggested relationships among the four taxa in the section. The results for section *Zea* reveal that *Z. mays* subsp. *huehuetenangensis* is the sister to *Z. mays* subsp. *mays* as judged by cpDNA sequence variation data, with *Z. mays* subsp. *parviglumis* appearing as a weakly supported sister group to these two taxa.

It is important in this context to emphazise that this data only represents the maternal inheritance and that when studying infra-specific taxa, the phylogenetic relationships are possibly obscured by the presence of gene flow and introgression among populations and subspecies, as indeed have been reported for the *Z. mays* complex by Ross-Ibarra et al. (2009). Therefore, we conclude that there was not enough sequence variation within the eight cpDNA regions used in this study to fully resolve the phylogenetic relationships among the five *Zea* species. Still, our results are in agreement with the idea that *Z. nicaraguensis* can be treated as a separate species distinct from *Z. luxurians*, and this is also in concordance with cytogenetic, morphological, and molecular data (Iltis and Benz, 2000; Buckler et al., 2006, Loaisiga et al., (unpublished), Ellneskog-Staam, 2007).

The Hardy–Weinberg equilibrium in Nicaraguan teosinte has not been always balanced due to many factors (mutations, non-random mating, gene flow and other), as is explained in Paper II. In addition it has been growing in isolated populations and under special conditions. As explained previously (4.2), *Z. nicaraguensis, Z. perennis* and *Z. diploperennis* showed high values in terms of allele richness. Warburton et al., (2011) found that there are shared alleles between other species of teosinte and maize which are not found in the *Z. mays* subsp. *parviglumis*. This does not conclusively demonstrate that gene flow has occurred directly from *Z. mays* subsp. *mexicana, Z. mays* subsp. *huehuetenangensis, Z. luxurians* or *Z. nicaraguensis*. These alleles may merely demonstrate the shared ancestry between the species, as all shared a common ancestor 150,000 years ago according to Ross-Ibarra et al. (2009).

According to Ross-Ibarra et al. (2009), there are different models to infer the evolution of a species. In the sympatric model, gene flow occurs during population divergence and is followed by eventual isolation between populations. Approximately half of the loci analyzed show evidence supporting introgression of sequence segments. Approximately 70% of these inferences point to recent introgression among subspecies of *Z. mays*, although there is a suggestion of gene flow between *Z. luxurians* and one or more subspecies of *Z. mays* in at least in six loci. Also, the estimate of ~55,000 years for the *Z. mays* subsp. *parviglumis*–maize divergence is nearly indistinguishable from the Mexicana divergence estimate of ~60,000 years, but several times higher than current dates for the domestication of maize (Pohl et al., 2007). The estimated timing of isolation for *Z. mays* subsp.

parviglumis–maize is more recent (~27,000 years), but still implausible given archeological records. Divergence between *Z. mays* subsp. *parviglumis*–maize and section *Luxuriantes* is estimated at ~140,000 years and followed by the cessation of gene flow at ~60,000 years ago.

Although we identify one possible recent introgression event between *Z. luxurians* and *Z. mays* subsp. *parviglumis*, strong support for the "sympatric model" suggests that gene flow between these taxa has been predominantly historical. This inference is consistent with current geographic patterns, because *Zea. luxurians* is isolated from known populations of either *Z. mays* subsp. *parviglumis* or *Z. mays* subsp. *mexicana* (Wilkes, 1977; Sanchez & Ruiz 1997; Fukunaga et al., 2005). Several lines of evidence nonetheless suggest that our inferred historical introgression is not implausible. Recent collections have identified populations of Z. *luxurians* in the Mexican state of Oaxaca (Cuevas, 2006), not far from the range of Mexicana. Furthermore, extant populations of a fourth subspecies of *Z. mays* subsp. *huehuetenangensis,* are currently found in Western Guatemala, suggesting that the ancestral ranges of *Z. mays* and *Z. luxurians* may have overlapped to some extent (Ross-Ibarra et al., 2009).

The present study supports this view by revealing *Zea* to be a genus with a relatively complex internal structure, as foreshadowed by Wilkes (1967), and one in which each population is genetically and morphologically sculptured to meet the demands of its particular environment (Doebley, 1983).

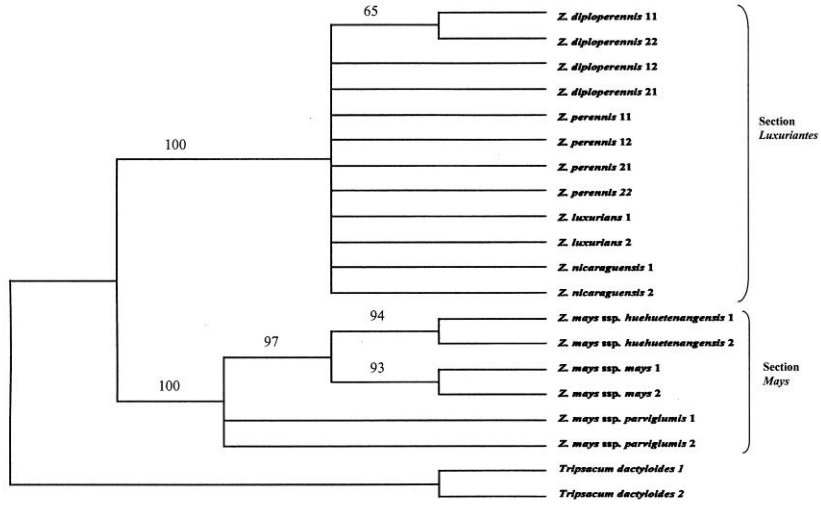

Figure 4. Strict consensus tree generated from sequences data from the eight cpDNA regions in nine *Zea* accessions using two *Tripsacum dactyloides* as out-group. Indels are treated as binary present/absent characters. Bootstrap support values are indicated on the branches (Full heuristic search; 1000 bootstrap replicates).

V. Conclusion

Based on the cytological, morphological, molecular and phylogenetic studies of teosintes from Meso-America, the following conclusions can be drawn:

1. *Zea luxurians* and *Zea nicaraguensis* are genetically more closely related to each other than to the rest of teosintes and C-banding pattern technique based cytogenetic analysis clearly supports that they are sister taxa.

2. Teosintes from Central America are more diverse than those in Mexican region, and the genetic variation within accessions is slightly higher than that among accessions in all analyzed taxa.

3. All populations of *Zea nicaraguensis* belong to the same taxon. The different populations are extremely threatened and the allele richness is high but many alleles are *rare* or *unique*.

4. Most of the total variation was explained mainly by six vegetative plant part characteristics and two tassel characteristics, according to principal component analysis. Also, several characteristics were associated principally with *Zea luxurians* and *Zea nicaraguensis* and to some degree with *Zea mays* subsp. *huehuetenangensis* as well, using different numerical taxonomic techniques.

5. Populations of *Zea nicaraguensis* revealed more rare and unique microsatellite alleles than *Zea luxurians*.

VI Recommendations and future prospects

1. To improve the resolution in the classification among Meso-American teosintes, it is recommended that more cpDNA regions should be used in combination with Internal Transcribed Spacer (ITS) and unlinked single-copy nuclear sequences in order to produce a better resolved phylogeny in *Zea*.

2. The significant genetic variations among teosinte populations from Nicaragua are an important signal for the conservation of all of those populations, with representative samples from each one in their natural habitats, through the implementation of a special management plan to permit the natural process of evolution of this important species.

3. Further research into *Zea nicaraguensis* is needed in order to find resistant or tolerant genotypes in relation to pests and diseases, and with a particular focus on the ability to cope with water-logged soil conditions as a source of germplasm for breeding.

4. In order to be prepared for the anticipated climate changes in the world it is urgently necessary to preserve and study all these species in order to find useful traits to incorporate in maize breeding.

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