ISSN 0719-8965

Molinai
Journal of Science

natural & agricultural sciences

Volume 2, Issue 2
July - December 2018

Universidad De Los Lagos

ISSN 0719-8965

Molinai
Journal of Science
natural & agricultural sciences

Volume 2, Issue 2
July - December 2018

www.molinai-journal.com
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Volume 2, Issue 2
July - December 2018

www.molinai-journal.com

ISSN 0719-8965

Molinai Journal of Science natural & agricultural sciences

RESEARCH PAPER

Journal home page: www.molinai-journal.com

A molecular phylogeny of the genus *Zea* based on cpDNA regions

Una filogenia molecular del género *Zea* basada en regiones de cpDNA

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How to cite this article:
Loáisiga, C.H., Díaz, O., Geleta, M. Rocha, O. y Quevedo R. (2018). A molecular phylogeny of the genus *Zea* based on cpDNA regions. J. sci. Molinai, Vol. 2, Issue 2: 26-41 (ISSN 0719-8965).

Abstract

Maize (*Zea mays* L.) domestication is inexorably linked to the development of New World cultures where maize remains a dominant food source. Maize's agricultural preeminence has led to its use as a model system for genetics and molecular biology. Despite the wealth of information obtained on this specie, the phylogeny of the genus *Zea* is still unresolved. Apart from cultivated maize the genus also includes the teosintes, i.e., wild subspecies of maize and four teosintes species, all distributed in the Meso-American region. Analysis of cpDNA variation has been proved to be one of the most effective methods for phylogenetic and evolutionary studies.

Phylogenetic relationships between nine teosinte accessions were evaluated based on eight non-coding regions of chroloplast DNA (psbZ-trnG, trnY-psbM, trnY-trnD, rps-16-trn-1, rps-16-trn-2, trnV-ndhC, ndhF-rpl32 and petA-psbJ). Parsimony analyses of combined sequence data from the cpDNA regions displayed strongly bootstrap supported trees with two major clades. In one clade, all accessions of *Z. perennis*, *Z. diploperennis*, *Z. luxurians* and *Z. nicaraguensis* appeared together and the other clade included *Z. mays* ssp. *huehuetenangensis*, *Z. mays* spp. *mays* and *Z. mays* spp. *parviglumis*. In conclusion, the trnY-trnD, trnY-psbD and rps16-trn1 intergenetic spacers regions proved to be useful for the phylogenetic analysis of *Zea*.

Key words: Teosinte, maize, genetic resources, chloroplasts, phylogeny

Resumen

La domesticación del maíz (*Zea mays* L.) está inexorablemente ligada al desarrollo de las culturas del Nuevo Mundo donde el maíz sigue siendo una fuente de alimento dominante. La preeminencia agrícola del maíz ha llevado a su uso como un sistema modelo para la genética y la biología molecular. A pesar de la gran cantidad de información obtenida sobre esta especie, la filogenia del género *Zea* sigue sin resolverse. Además del maíz cultivado, el género también incluye las teosintes, es decir, subespecies silvestres de maíz y cuatro especies de teosintes, todas distribuidas en la región mesoamericana. El análisis de la variación de cpDNA ha demostrado ser uno de los métodos más efectivos para los estudios filogenéticos y evolutivos.

Las relaciones filogenéticas entre nueve accesiones de teocinte se evaluaron en base a ocho regiones no codificantes de ADN cloroplastidial (psbZ-trnG, trnY-psbM, trnY-trnD, rps-16-trn-1, rps-16-trn-2, trnV-ndhC, ndhF-rpl32 y petA-psbJ). Los análisis de parsimonia de datos de secuencia combinados de las regiones de cpDNA mostraron árboles compatibles con bootstrap con dos clados principales. En un clado, todas las accesiones de *Z. perennis*, *Z. diploperennis*, *Z. luxurians* y *Z. nicaraguensis* aparecieron juntas y el otro clado incluía *Z. mays* ssp. *huehuetenangensis*, *Z. mays* spp. *mays* y *Z. mays* spp. *parviglumis* En conclusión, las regiones de espaciadores intergenéticos trnY-trnD, trnY-psbD y rps16-trn1 demostraron ser útiles para el análisis filogenético de *Zea*.

Palabras clave: Teosinte, maíz, recurso genético, cloroplastos, filogenia

Introduction

Ecological and phylogenetic data provide heterogeneous information about the origin, evolutionary history and present distribution of species and species groups (Wiens, 2004). During the last decade an increasing number of studies have combined different types of information to test biogeographical (Leathwick, 1998), ecological (Anderson *et al*., 2002a) and evolutionary hypotheses (Graham *et al*., 2004). Integrated research has revealed new insights about the factors influencing the evolution and distribution of species and have become powerful tools for addressing issues in evolution, biogeography, ecology and conservation biology (Peterson, 2001). DNA analysis has been proven to be one of the most effective molecular methods for studies of phylogeny and evolution of different plant species.

The nuclear genome is inherited in a biparental manner whereas the chloroplast and mitochondrial genome are generally inherited maternally. Biparental inheritance and polyploidy in the nuclear genome may cause difficulties for evolutionary analysis, whereas analyses of chloroplast genomes are effective in the investigation of relationships in plants because of their maternal inheritance (Tomotaro *et al*., 2002).

Maize (*Zea mays* L.) domestication is inexorably linked to the development of New World cultures and maize remains a dominant food source. Maize's agricultural preeminence has led to its use as a model system for genetics and molecular biology (Doebley, 1990a; Kellogg & Birchler, 1993). Despite the wealth of information obtained on *Zea mays*, the phylogeny of the genus *Zea* is still unresolved.

Apart from cultivated maize (*Zea*, *mays* ssp. *mays*), the genus also includes wild subspecies of maize and four wild species. All wild taxa are collectively referred to as teosintes. Despite being a relatively small genus it has been divided into two sections, i.e., section *Zea* and section *luxuriantes*. According to Doebley (1990b) the former section includes *Z. mays* ssp. *mays* (cultivated maize), *Z. mays* ssp. *mexicana* (Schrad.) Iltis (a large-spikeleted teosinte adapted to the drier high elevation (ca. 1600 – 2700 m) of northern and central Mexico, *Z. mays* ssp. *parviglumis* Iltis & J.F. Doebley (a small spikelet teosinte adapted to the moister middle elevation (ca. 400 – 1800 m) of south-western Mexico) and *Z. mays* ssp *huehuetenangensis* Iltis & J.F. Doebley (an annual teosinte found only in the province of Huehuetenango in western Guatemala).

Section luxuriantes includes *Z. luxurians*, Durie & R.M. Bird (a diploid annual teosinte from Central America), *Z. diploperennis* Iltis, J.F Doebley & R. Guzman (a diploid perennial teosinte from Jalisco, Mexico), *Z. perennis* (Hitchc) Reevies & Mangelsd (a tetrapolid perennial teosinte from Jalisco, Mexico) and *Z. nicaraguensis* Iltis and B. F. Benz (an annual diploid teosinte from Nicaragua) (Iltis & Benz, 2000).

Most of the teosintes taxa have thus narrow geographic distributions, often consisting of only a few local populations. The exceptions are *Z. mays* ssp. *mexicana* and *Z. mays* ssp. *parviglumis*, which are widely distributed in Mexico (Fukunaga *et al*., 2005). The status of *Z. luxurians* has been questioned by Fukunaga *et al*. (2005). They also treat it as a "geographical group" of *Z. luxurians* without ascribing it any rank.

Chloroplast restriction site, isozyme and cytogenetic analyses (Kato, 1976; Doebley & Goodman, 1984; Renfroe & Blanton, 1987) have established that maize (*Z. mays* ssp. *mays*) was domesticated from *Z. mays* populations in Central Mexico (Doebley, 1990a). The conclusions drawn from those molecular studies were incorporated in the current classification of *Zea* (Doebley, 1990a), but these studies did not resolve the detailed phylogeny of the genus.

The chloroplast genome is useful in providing information on the evolutionary patterns and processes in plants (Raubeson & Jansen, 2005). Chloroplast DNA (cpDNA) sequence variations, either individual or combined with other genomes, have been widely used for inferring phylogenetic relationships of different taxa, including *Hordeum*, *Triticum* and *Aegilops* (Gielle & Taberlet, 1994) and *Sorghum* (Dillon *et al*., 2007). Compared to coding regions, the noncoding chloroplast regions are phylogenetically more informative at lower taxonomic levels because they are under less functional constraints and evolve rapidly (Gielle & Taberlet, 1994). Of these regions, trnT-trnL was reported to be one of the regions that provides the highest number of potentially informative characters across all polygenetic lineages and possesses enough phylogenetic signals for studies at lower taxonomic levels (Shaw *et al*. 2005).

The objectives of the present study were: (1) to evaluate whether the eight non -coding regions of cpDNA could give an improved picture of the phylogeny among the taxa in the genus *Zea*; (2) to study whether cpDNA data supports the current sectional delineation in the genus *Zea*; and (3) to study the relationships between *Z. nicaraguensis* and *Z. luxurians*.

Material and Methods

Study material

Seeds of all known species of *Zea* were provided by the CIMMYT Gene Bank in Mexico and the National Gene Bank (REGEN) of Nicaragua. In total nine accessions belonging to five different species, one of which is represented by three subspecies, were included in the study, namely, *Zea diploperennis*, *Zea perennis*, *Zea luxurians*, *Z. nicaraguensis*, *Zea mays* ssp. *mays*, *Zea mays* ssp. *huehuetenanagensis* and *Zea mays* ssp. *parviglumis*. Each taxon was represented by one accession, except *Z. diploperennis* and *Z. perennis*, which were represented by two accessions. For each accession, two individuals were analyzed. Two accessions of *Tripsaum dactyloides* and one accession of *Sorghum bicolor* were used as outgroups. Information on accession numbers and origin is provided (Table 1).

Table 1. Geographic information for the accessions of Mesoamerican teosintes used in this study.

Accession	Origin	Taxon	Altitude (MASL)	LAT	LONG
9476	Mexico	*Zea diploperennis*-1	1950	19°35'	104°12'
10003	Mexico	*Zea diploperennis*-2	1900	19°35'	104°16'
8837	Mexico	*Zea perennis*-1	940	19°38'	103°34'
9475	Mexico	*Zea perennis*-2	1600	19°42'	103°26'
9478	Guatemala	*Zea luxurians*	800	14°38'	89°38'
4290	Nicaragua	*Zea nicaraguensis*	10	12°53'	86°58'
9479	Guatemala	*Zea mays* ssp. *Huehuetenangensis*	1300	15°40'	91°45'
9477	Mexico	*Zea mays* ssp. *parviglumis*	850	19°49'	104°11'
CV NB-6	Nicaragua	*Z. mays* ssp. *Mays*	100	12°53'	86°58'
MIA 34536	Venezuela	*Tripsacum dactyloides* var. *meridonale*-1	2200	8°28'	71°34'
MIA 35920	Colombia	*Tripsacum dactyloides* var. *meridonale*-2	1300	27°25'	80°20'
ZMB-7204	Zambia	*Sorghum bicolor*	600	15°27'	28°17'

MASL: metres above sea level; LAT: latitude; LONG: longitude

DNA extraction, PCR and sequencing

DNA was extracted from fresh leaf tissue of seedlings approximately two weeks of age using a modified Arabidopsis extraction method. The quality of the DNA was analyzed by agarose gel electrophoresis and DNA concentration was determined using a Nanodrop ® ND-1000 spectrophotometer (Saveen Werner, Sweden).

The primers for the amplification and sequencing of the psbZ-trnG, trnY-psbM, trnY-trnD, rps-16-trn-1, rps-16- trn-2, trnV-ndhC, ndhF-rpl32 and petA-psbJ regions were specially designed for this study while the trnT-trnL region was amplified and sequenced using the universal primers designed by Taberlet *et al*. (1991). A primer pair was used for each of the cpDNA regions. However, two primer pairs were designed for the amplification of the trnY-psbM region.

The sequences of the primers and information on specific primers were supplied by Eurofins MWG GmbH (Table 2). A Gene AMP PCR system 9700 thermocycler was used for amplification, where the initial denaturation was carried out at 94° C for 3 minutes followed by 30 cycles of 94° C for one minute, 48° C for one minute, 72° C for two minutes and the final extension at 72° C for 7 minutes. Prior to purification, PCR products were run on 2% agarose gels to confirm amplification of the targeted region.

Successfully amplified samples were purified using the QIAquick PCR purification kit (Qiagen GmbH, Germany) and the micro centrifuge according to the manufacturer's instructions. Nine microliters of purified PCR products were mixed with 1 µl of sequencing primers and sent to the sequencing facility in the University of Oslo, Norway (http://www.bio.uio.no/ABI-lab/), where the DNA sequencing was done.

Sequence alignment and data analysis

The quality of the sequences was visually inspected using Sequence Scanner version 1.0 (Applied Biosystems). Multiple sequence alignment was performed using ClustalX version 2.1.10 (Larkin *et al*. 1999) and checked by eye. The sequences were edited using BioEdit version 7.0.9 (Hall 1999). The analysis of the combined data sets were made using cladistic parsimony and all characters were unordered and equally weighted with PAUP* 4.0 b10 (Swofford, 2002). Heuristic searches were carried out using TBR branch swapping and simple sequence stepwise additions.

Table 2. Primers used to amplify and sequence the five non-coding regions of cpDNA.

Region of cpDNA	Primer name[1]	Primer sequence (5'→3')
psbZ-trnG	tnSM–fw	TGC TTC TCC TGA TGG TTG GT
psbZ-trnG	tnSM–rv	GCT CGC TAC ATT GAA CTA CGC
trnYa-psbM	trYB–fw	GGT TAA TGG GGA CGG ACT G
trnYa-psbM	trYB–rv	AGG AAG TTA AGA TGA GGG TGG T
trnYb-psbM	psBD–fw	CTG TCA AGG CGG AAG CTG
trnYb-psbM	psBD–rv	GGG TCA CAT AGA CAT CCC AAT
trnY-trnD	trTD–fw	TGA CGA TAT GCT TAC GCT GGT T
trnY-trnD	trTD rv	AAT CCC TGC GGG GTG TAT
Rps16-trnQ	rps16-trnQ-1-fw	TTTATCGGGGAACACTCTGC
Rps16-trnQ	rps16-trnQ-1-rv	CGATCTCGATCTGTGATTCTTT
trnV-ndhC	trnV-ndhC-fw	CTCAAACCAAAGGCAAGGAG
trnV-ndhC	trnV-ndhC-rv	TTCGAATCCGTATAGCCCTAA
ndhF-rp	ndhF-rp132-fw	TAACTGGAAGTGGGAGAAGAGG
ndhF-rp	ndhF-rp132-rv	TTGGAACTGCCATTCAAAAAG
peta-psbJ	petA-psbJ-fw	CCTGACGGTAGCAAGAGTAACA
peta-psbJ	petA-psbJ-rv	TAAAAATCAAAACCACCCTTCC

[1] All primers were used for both PCR amplification and sequencing

Sequence alignment gaps were treated as missing data and the 35 indels were recorded as binary (present/absent) characters. The analyses were used to generate strict consensus trees that were evaluated for phylogenetic information. In order to estimate node support, 1,000 bootstrap replicates were performed PAUP* 4.0 b10 (Swofford, 2002). Uninformative characters were excluded in the calculation of consistency index (CI), retention index (RI) and rescaled consistence index (RC). *Tripsacum dactyloides* or *Sorghum bicolor* were used as outgroups in different analyses.

Results and Discussion

The combined eight chloroplast regions of 2312 bp together with the 35 indels gave a data matrix of 2347 characters. Of these, 2214 characters were constant and 133 were variable, of which only 57 were parsimony informative. The result from the heuristic search with *S. bicolor* as the outgroup resulted in 12 equally parsimonious trees 147 steps long (CI=0.82, RI=0.92 and RC=0.84), with six of the nine branches having bootstrap support values above 90 per cent (Fig. 1).

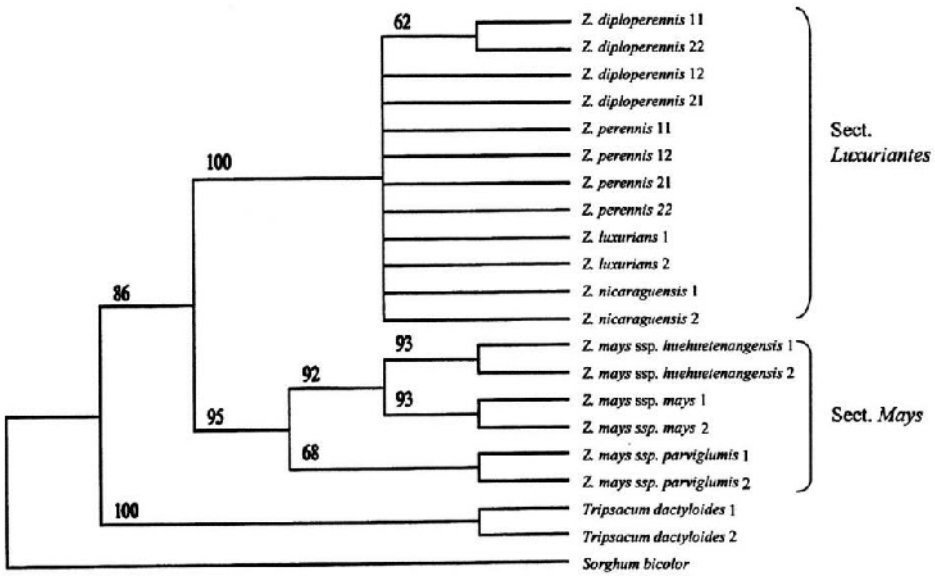

Figure 1. Maximum parsimony 50% majority rule consensus tree (1000 boot-strapping replicates with 100 random additions; MaxTrees=100) generated from a phylogenetic analysis of DNA sequences data from the eight regions of cpDNA of 8 teosinte accessions, *Z. mays*, *S. bicolor* and *T. dactiloides* as an outgroup species. Indels are treated as missing data. Clades are indicated by a letter below the branches and bootstrapping values of > 50 % are indicated on the branches.

A strict consensus tree with the addition of 148 steps long trees (n=5,886) generated an identical tree except for the collapse of the weakly supported clade of two *Z. diploperennis* sequences.

In order to collapse more branches, trees up to 150 steps long had to be included (n>500,000). Then, nearly all internal structures collapsed in the ingroup and only two clades remained, i.e., a *Zea* clade and a *Tripsacum* clade. This demonstrates that *Zea* and its supposed sister-group *Tripsacum* belong to different clades and *Tripsacum* can be used as an outgroup to *Zea*.

The result from the heuristic search excluding *S. bicolor* data and using *T. dactyloides* as the outgroup resulted in 24 equally parsimonious trees 87 steps long (CI=0.92, RI=0.97 and RC=0.91), with five out of six ingroup branches having bootstrap support values above 90 per cent (Fig. 2).

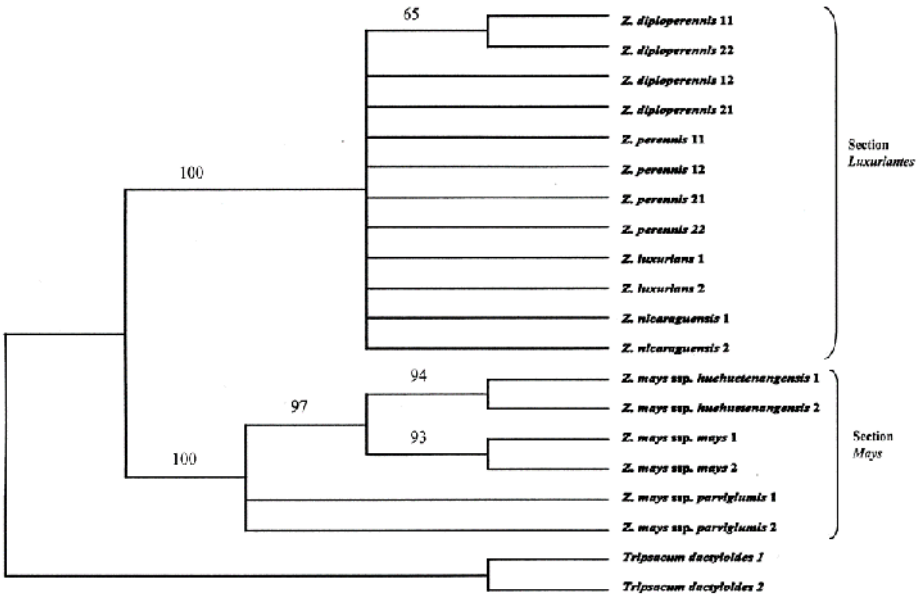

Figure 2. Strict consensus tree generated from sequences data from the eight cpDNA regions in nine Zea accessions using two Tripsacum dactyloides as outgroup. Indels are treated as binary present/absent characters. Bootstrap support values are indicated on the branches (Full heuristic search; 1000 bootstrap replicates).

A strict consensus tree with the addition of 88 steps long trees (n=11,760) generated an identical tree, again with the exception that the clade of two *Z. diploperennis* sequences collapsed.

Sequences characteristics of teosinte accessions

The sequences characteristics and parsimony-based tree statistics of eight non-coding regions of cpDNA are summarized in Table 3. The alignment sequences derived from all cpDNA regions revealed differences in sequences length between teosinte accessions. The longest sequences were obtained from Rps16-trn2 and ndhF- rp132. By contrast, the Rps16-trn2 spacer provided the shortest sequences.

Table 3. Sequence characteristics and tree statistics of the cpDNA regions, from maximum parsimony analysis (MPA).

Region of cpDNA	psbZ-trnG	trnYa-psbM	trnYb-psbM	trnY-trnD	Rps16-trnQ	trnV-ndhC	ndhF-rp	petA-psbJ	Average
LAS	297	305	420	261	126	273	368	262	289
PIC	2 (0.67%)	7 (2.3%)	16 (3.9%)	6 (2.3%)	1 (0.8%)	1 (0.37%)	2 (1.1%)	2 (0.76%)	4.62 (1.5%)
TL	6	16	30	13	3	10	23	2	12.8
CI	1.00	1.00	0.867	1.00	1.00	1.00	1.00	1.00	0.983
HI	0.00	0.00	0.20	0.00	0.00	0.00	0.00	0.00	0.025
RI	1.00	1.00	0.886	1.00	1.00	1.00	1.00	1.00	0.985
RCI	1.00	1.00	0.768	1.00	1.00	1.00	0.00	0.00	0.725

LAS: length of aligned sequences; PICs: parsimony-informative characters (number and per cent); TL: tree length; CI: consistency index; HI: homoplasy index; RI: retention index; RC: rescaling consistency index.

The genetic analysis of teosinte at the intra-accession levels was based on nine polymorphic loci. The number of haplotypes per accession ranged from zero to four. No DNA sequences variation was obtained in the *Z. diploperennis2*, *Z. perennis1* and *Z. nicaraguensis*.

Comparative DNA sequencing has become a widespread tool for inferring phylogenetic relationships as it is relatively fast and convenient. Phylogenetic elucidation of evolutionary processes that generate biological diversity have been accomplished even at lower taxonomic levels using a non-coding region of chloroplast genome and ITS of the nrDNA (Mort *et al*., 2007; Kårehed *et al*., 2008).

The chloroplast genome is believed to essentially display only maternal inheritance in the majority of angiosperms (Mogensen, 1996; Keeling, 2004; Udall & Wendel, 2006). Systematic treatments of Zea are usually based on evidences from several biological disciplines, e.g., isozyme, chloroplast restriction site, mitochondrial, chromosomal knob and nuclear ribosomal marker studies (Kato, 1976; Smith *et al*., 1982; Doebley *et al*., 1984; Doebley *et al*., 1987a; Allen, 1992; Buckler & Holtsford, 1996), however, hitherto not by cpDNA sequence variation.

In the present study, not all of the eight cpDNA region primers successfully amplified the target region in the teosinte accessions. The results revealed a small number of parsimony informative variable sites across all DNA regions; nevertheless the trnY-trnD, trnY-psbD and rps16-trn1 may support the phylogenetic analysis of the genus *Zea*.

Various phenetic and phylogentic studies have consistently supported a differentiation amongst the taxa of *Zea*. The two perennial species (*Z. perennis* and *Z. diploperennis*) generally form a cluster/clade, with *Z. luxurians* branching out next to them. Although this relationship is clearly supported by cpDNA restriction site variation (Doebley *et al*., 1987a), it is neither supported nor contradicted by the tree produced by nuclear ribsosomal markers (Buckler & Holtsford, 1996; Doebley *et al*., 1984). Buckler *et al*. (2006), in research based on isozyme data, found that *Z. nicaraguensis* appears to be allied with *Z. luxurians*, but it is at the same time consistently distinct from this species, hence supporting Iltis & Benz's (2000) conclusion that "*Z. luxurians*" from Nicaragua is better treated as an independent species. However, Fukunaga *et al*. (2005), in a study of 93 microsatellite loci, found that *Z. nicaraguensis* appeared nested within the *Z. luxurians* clade. Thus, they concluded that "*nicaraguensis*" could possibly be given the rank of a subspecies of *Z. luxurians*, but state that more data is needed.

Our data strongly supports a clade consisting of *Z. perennis*, *Z. diploperennis*, *Z. luxurians* and *Z. nicaraguensis* but unfortunately without any resolution within this clade, since all species appear as a basic unresolved polytopy. Thus, our data can neither support nor refute any of the previously suggested relationships among the four taxa. There is not enough differentiation within the eight cpDNA regions to resolve the phylogeny among these closely related species. Ng'uni *et al*. (2010) obtained good resolution using ITS sequences in S. bicolor germplasm; for this reason it might be interesting to test ITS sequences in future work with teosinte genera.

The other strongly supported clade in our data consists of *Z. mays* sensu lato and this is in agreement with the current classification of the genus (Doebley, 1990b). However, the relationships within *Z. mays* (i.e., among subspecies) are still unclear. Unfortunately *Z. mays* ssp. *mexicana* was not available for inclusion in this study. Still, our results reveal that *Z. mays* ssp. *huehuetenangensis* is the sister to *Z. mays* ssp. *mays* as judged by cpDNA sequence variation data, with *Z. mays* ssp. *parviglumis* appearing as a weakly supported sister group to these two taxa.

However, in this context it is important to remember 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). This could explain the contradictory results regarding affinities within the *Z. mays* complex. For example, data suggests that *Z. mays* ssp. *huehuetenangensis* is strongly divergent from the other subspecies, which are much more similar to each other (Doebley, 1990a).

Conclusion

In conclusion, the trnY-trnD, trnY-psbD and rps16-trn1 intergenetic spacers regions were useful to support the phylogenetic analysis of the genus *Zea*. There was not however enough differentiation within the eight cpDNA regions to resolve the phylogeny among these closely related species in section Luxuriantes. Other molecular studies are needed to complete clarify the questions.

Acknowledgments
Carlos Henry Loáisiga thanks the Swedish International Development Agency (SIDA/SAREC) for funding part of this work and the PhD Programme in the National Agrarian University in Nicaragua for providing experimental facilities.

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Received: Septembre 12, 2018
Accepted: December 20, 2018