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Genetic Diversity Analysis of *Heliconia psittacorum* Cultivars and Interspecific Hybrids Using Nuclear and Chloroplast DNA Regions

Walma Nogueira Ramos Guimarães[1,*], Gabriela de Morais Guerra Ferraz[1], Luiza Suely Semen Martins[2], Luciane Vilela Resende[3], Helio Almeida Burity[4] and Vivian Loges[1]

[1]*Department of Agronomy, Federal Rural University of Pernambuco, Recife, Pernambuco,*
[2]*Department of Biology, Biochemical Genetics Laboratory/Genome, Federal Rural University of Pernambuco,*
[3]*Department of Agronomy, Federal University of Lavras, Minas Gerais,*
[4]*Agronomic Research Institute of Pernambuco, Recife, Pernambuco, Brazil*

1. Introduction

Heliconia cultivation has intensified in Brazil as a cut flower, especially in the Northeast region. This ornamental rhizomatous herbaceous plant from the *Heliconia* genus, belongs to the Musaceae family, now constitutes the Heliconiaceae family in the Zingiberales order. The various species of *Heliconia* are subdivided into five subgenera: *Heliconia, Taeniostrobus, Stenochlamys, Heliconiopsis* and *Griggisia*; and 28 sections (Kress *et al*., 1993). In *Heliconia* genus, the number of species ranges from 120 to 257 and there are also a great number of cultivars and 23 natural hybrids (Berry and Kress, 1991; Castro *et al*., 2007), these plants can be found either in shaded places, such as forests or at full Sun areas, such as forest edges and roadsides (Castro and Graziano, 1997). They are native from Tropical America (Berry and Kress, 1991), found at different altitudes, from sea level up to 2.000 meters in Central and South America, and up to 500 meters in the South Pacific Islands (Criley and Broschat, 1992).

Heliconia hybrids comprise many of the major cultivars as cut flowers, like *H. psittacorum* x *H. spathocircinata* cv. Golden Torch, cv. Golden Torch Adrian, cv. Alan Carle and *H. caribaea* x *H. bihai* cv. Carib Flame, cv. Jacquinii, cv. Richmond Red (Berry and Kress, 1991). Many heliconia species are identified through their morphological differences, such as the size and color of its flowers and bracts. These characteristics can be influenced either by geographic isolation or by environmental factors, such as light and nutrients (Kumar *et al*., 1998). *H. psittacorum* clones, even when closely grown, can vary in blooming, size and color of bracts, as well as post harvest durability (Donselman and Broschat, 1986).

*Corresponding author

The natural variation among heliconia individuals or populations has led to taxonomic identification doubts among farmers and researchers. Thus, genebanks have played an important role in genetic diversity conservation, providing raw material for crop breeding, including landraces and their wild relatives. DNA markers, which allow the access to variability at DNA level, emerge as an efficient alternative for plant species characterization by quantifying diversity and determining its genetic structure (Bruns *et al*., 1991).

The choice on which molecular marker technique shall be used depends on its reproducibility and simplicity. Kumar *et al*. (1998) distinguished three cultivars of the hybrid *H. psittacorum* x *H. spathocircinata* cvs. Golden Torch, Red Torch and Alan Carle which showed only slight differences in RAPD markers profile from *H.* x *nickeriensis* Maas and de Rooij (*H. psittacorum* x *H. marginata*), they also observed similarities in RAPD profiles and morphology. The authors concluded that two triploid *H. psittacorum*: cv. Iris and Petra, are supposed to be the same genotype.

Genetic diversity studies grew up in interest during the last years (Jatoi *et al*., 2008; Kladmook *et al*., 2010). As a result, nucleotide sequences of ribosomal genes (rDNA) and chloroplast genes (cpDNA) have been exploited to investigate several individuals of the Zingiberales order (Kress, 1990, 1995; Kress *et al*., 2001) once they are not capable of lateral transfers and are not subject to the same functional limitations, they allow greater confidence in the results (Camara, 2008). The unit of ribosomal eukaryotic organisms consists of three genic and three non-genic regions. On one hand, the genic regions (18S, 5,8S and 26S) are conserved and evolve slowly. On the other hand, non-genic regions, known as ITS - Internal Transcribed Spacer (ITS-1 and ITS-2), evolve rapidly, showing high polymorphism and, therefore, allowing its use at higher hierarchical levels. The variability found in these regions could be the result of mutations in these areas, since they suffer less selection pressure and may be well used to study genetic diversity in plants (Bruns *et al*., 1991).

This molecular marker is important from the genetic variability assessment point of view, because the rDNA mutltigenic family once subjected to a rapid evolution in concert event, allows greater precision in the reconstruction process of the relationship between species based on sequencing, since this phenomenon increases the intragenomic uniformity (Baldwin *et al*., 1995). These authors also affirm that due to the biparental inheritance of the nuclear genome it is possible to study the origin of hybrids and their parents. Moreover, chloroplast genes (cpDNA), such as the leucyn and fenilalanyn of RNA transporter (*trnL-trnF*), the treonyn and leucyn of RNA transporter (*trnT-trnL*) and the protein small 4 (*rps4*), have been used successfully to solve genetic diversity doubts in taxonomic lower levels. Johansen (2005), for example, studying the genetic diversity in Zingiberales order, using cpDNA, has positioned all Heliconiaceae and Musaceae within a same clade.

The aim of this study was to evaluate genetic diversity involving *Heliconia psittacorum* cultivars and interspecific hybrids of the Federal Rural University of Pernambuco *Heliconia* Germplasm Collection (UFRPE-HCG), using nuclear and chloroplast DNA regions.

2. Materials and methods

2.1 Plant material and genomic DNA extraction

The *Heliconia* Germplasm Collection (UFRPE-HCG) is located in Camaragibe-PE at 8°1'19'' South, 34º59'33'' West and 100 m above the sea level, in a 0.3 ha experimental area. The

average annual temperature is 25.1ºC and monthly rainfall of 176 mm, with maximum of 377 mm and minimum of 37 mm (ITEP, 2008). This study evaluated 11 *Heliconia psittacorum* cultivars and interspecific hybrids (Table 1) obtained by exchange with research institutions and farmers from the states of Pernambuco (PE), Alagoas (AL) and Sao Paulo (SP) in Brazil. The analyzed genotypes presented short size, musoid habit and erect inflorescence disposed at a single plan (Berry and Kress, 1991).

Genotypes[a]	Location	Subgenus and Section[b]	Description[c]
Hybrids			
H. x *nickeriensis Maas and de Rooij* (*H. marginata* x *H. psittacorum*)	Paulista - PE	*Heliconia* *Pendulae*	BC- yellow-orange; OV- dark yellow distally, light yellow proximally, PD- yellow, SE- dark yellow
H. psittacorum L.f x *H. spathorcircinata* Aristeguieta cv. Golden Torch Adrian	Paulista - PE	*uncertain*	BC- yellow-Red; OV- yellow, PD- yellow with indistinct blackish green area distally, SE- dark yellow
H. psittacorum L.f x *H. spathorcircinata* cv. Golden Torch	Paulista - PE	*uncertain*	BC- yellow; OV- yellow, PD- yellow with indistinct blackish green area distally, SE- dark yellow
H. psittacorum L.f. x *H. spathocircinata* cv. Red Opal	Paulista - PE	*uncertain*	BC- orange; OV- yellow, PD- yellow, SE- dark yellow with indistinct blackish green area distally
Triploid[d]			
H. psittacorum L.f. cv. Suriname Sassy	Paulista - PE	*Stenochlamys* *Stenochlamys*	BC- pink-green; OV- orange distally, yellow proximally, PD- orange to cream, SE- orange with indistinct blackish green area distally
H. psittacorum L.f. cv. Sassy	Paulista - PE	*Stenochlamys* *Stenochlamys*	BC- pink-green; OV- orange distally, yellow proximally, PD- yellow green, SE- orange with indistinct blackish green area distally
Heliconia sp. (suposed to be *H. psittacorum* cv. Sassy)	Maceió - AL	*Stenochlamys* *Stenochlamys*	BC- pink-lilac; OV- green distally and yellow green proximally, PD- yellow green, SE- orange with indistinct blackish green area distally
Suposed triploid			
H. psittacorum L.f .cv. Strawberries and Cream	Paulista - PE	*Stenochlamys* *Stenochlamys*	BC- pink-yellow; OV- yellow to cream, PD- cream, SE- pale yellow with green spot on distally corner
H. psittacorum L.f. cv. Lady Di	Ubatuba - SP	*Stenochlamys* *Stenochlamys*	BC- red; OV- yellow, PD- light yellow to cream, SE- light yellow with distally dark green band and white tip
H. psittacorum L.f. cv. St. Vincent Red	Ubatuba - SP	*Stenochlamys* *Stenochlamys*	BC- red-orange; OV-orange distally, orange to cream proximally, PD- orange, SE- orange with indistinct blackish green area distally
H. psittacorum L.f. cv. Red Gold	Paulista - PE	*Stenochlamys* *Stenochlamys*	BC- red-orange; OV- yellow, PD- yellow, SE- dark yellow with indistinct blackish green area distally

[a]Identification based on Berry and Kress (1991) and Castro *et al*. (2007); [b]Based on Kress *et al*. (1993); [c]BC: bract color; OV: ovary; PD: pedicel; SE: sepals. [d]Ploidy (Costa *et al*., 2008).

Table 1. Genotypes, location, classification and description for 11 *Heliconia psittacorum* cultivars and interspecific hybrids of the UFRPE Heliconia Germplasm Collection used in this study

Molecular markers analyses occurred in the Plant Biotechnology Laboratory - UFRPE. The optimization of the DNA extraction protocol was performed using fresh young leaves samples of heliconia, harvested in the earliest stage of development and treated under three conditions: harvested, packed in a polystyrene box containing liquid nitrogen and taken to the Laboratory for immediate DNA extraction; harvested and frozen at -20°C for 1 day before extraction; harvest and preserved in silica gel for 5 days before extraction.

In the DNA extraction, Doyle and Doyle (1990) protocol were used with modifications, which was prepared at a 2x CTAB (hexadecyltrimethylammonium bromide) buffer solution. It was added 700 microliter extraction buffer to 200 mg of macerated leaves in test tubes and taken to bath at 65°C. The tubes, after cooled at room temperature, were centrifuged and the supernatant transferred to new tubes. Supernatant was added to 700 microliter (μL) CIA (Chloroform-Isoamyl Alcohol) and then centrifuged was performed.

The supernatant was added to 700 microliter (μL) CIA (Chloroform-Isoamyl Alcohol) and then centrifuged. After this process, supernatant was added to 500 μL of cold isopropanol and stored for 24 hours in a freezer at -20ºC. Subsequently, it was washed twice with 70% ethanol and with 95% ethanol. The precipitate was dried at room temperature for 20 minutes and then resuspended with 300 μL TE containing RNAse, incubated at 37°C for 30 minutes, then 5M NaCl and 300 μL of cooled isopropanol were added, in which the DNA was precipitated. Solution was incubated at 4°C throughout the night and the pellet resuspended in 300 μL TE. The DNA was quantified in 0.8% agarose gel.

Primers	Sequence	Number of Basis
ITS 1	5′- TCCGTAGGTGAACCTGCGG -3′	19
ITS 2	5′-GCTGCGTTCTTCATCGATGC-3′	20
ITS 3	5′-GCATCGATGAAGAACGCAGC -3′	20
ITS 4	5′-TCCTCCGCTTATTGATATGC-3′	20
ITS 5	5´-GGAAGTAAAAGTCGTAACAAGG -3´	22
EF11	5-GTGGGGCATTTACCCCGCC-3'	19
EF22	5´-AGGAACCCTTACCGAGCTC-3´	19
trnL	5´-GGTTCAAGTCCCTCTATCCC -3´	20
trnF	5´-ATTTGAACTGGTGACACGAG-3´	20
trnS	5´-TACCGAGGGTTCGAATC -3´	17
rps5′	5´-ATGTCCCGTTATCGAGGACCT -3´	21
rps3′	5′ –ATATTCTACAACTAACAACTC – 3′	21

Table 2. Sequence of primers used in amplification reactions in genotypes of the UFRPE Heliconia Germplasm Collection used in this study

A set of 12 primers (Table 2) and 10 combinations of this primers were selected and tested for the ITS analysis: ITS1-ITS4; ITS5-ITS4; ITS1-ITS2; ITS5-ITS2; ITS3-ITS4 based on White *et*

al. (1990); and EF11-EF22; and for chloroplast genes analysis: *rps3'-rps5'* (Sanchez-Baracaldo, 2004)*; trnL-trnF* (Sang *et al*., 1997); *trnS-trnF* and *trnS-trnL.*

2.2 PCR amplification

The DNA amplification using PCR was performed to a final volume of 25 μL containing 1 μL template DNA, 0.3 μL Taq-DNA polymerase (Invitrogen), 2.51 μL Tris-HCl (pH 8, 0), and 0.75 μL $MgCl_2$, 2 μL of each dNTPs, 1 μL primer, 1 μL oligonucleotide 1 and 2; and 15.45 μL milli-Q water to complete the reaction.

Amplifications were performed in a thermocycler MJ Reseach, Inc., PTC100 under the following conditions: step 1 - following a denaturation step of 95°C for 3 minutes; step 2 – 94°C for 1 minute; step 3 – 58°C for 1 minute for annealing temperature; step 4 – 72°C for 1 min (repeat steps 2/3/4 for 29 cycles) followed by a final extension at 72°C for 10 minutes and 10°C for 24h. The PCR product visualization was performed in 1.5% agarose gel stained with SYBER Gold (Invitrogen), visualized under ultraviolet light and recorded on a digital Vilber Lourmat photographer.

2.3 Statistical analysis

Through the interpretation of gels, molecular data were tabulated as presence (1) or absence (0) of DNA fragments by primers for each genotype. Genetic similarities among genotypes were determined based on the Jaccard (1908) coefficients. A dendrogram was then constructed using the unweighted pair-group method of the arithmetic average (UPGMA) based on the similarity matrix. The cluster analyses were conducted using the computer program Gene (Cruz, 2006).

3. Results

The best condition for heliconia DNA extraction was using leaves in the earlyest stage of development, harvested, packed in a polystyrene box containing liquid nitrogen and taken to the Laboratory for immediate DNA extraction.

3.1 Primers selection

Primer combination ITS4-ITS3 resulted in most of the polymorphic band region, while for the primer combination ITS4-ITS5 it was observed the least polymorphism. The primers used amplified from 1 to 6 band regions, with clear polymorphism between the genotypes. The amplifications of the nuclear region that includes the spacers ITS1-ITS2 and EF11-EF22 (Fig. 1) generated fragments of approximately 396 to 506 pb, which agrees with Baldwin *et al*. (1995), by claiming that ITS markers have numerous small sized copies, reaching up to 700 pb.

Chloroplast regions amplifications that used the primers tRNA of leucine and phenylalanine (*trnL-trnF*) generated fragments of approximately 1636 pb (Fig. 2). For the spacers regions *rps3'-rps5'* as well as for the regions *trnS-trnL* and *trnS-trnF*, it was observed monomorphic and polymorphic band patterns for the evaluated cultivars and hybrids.

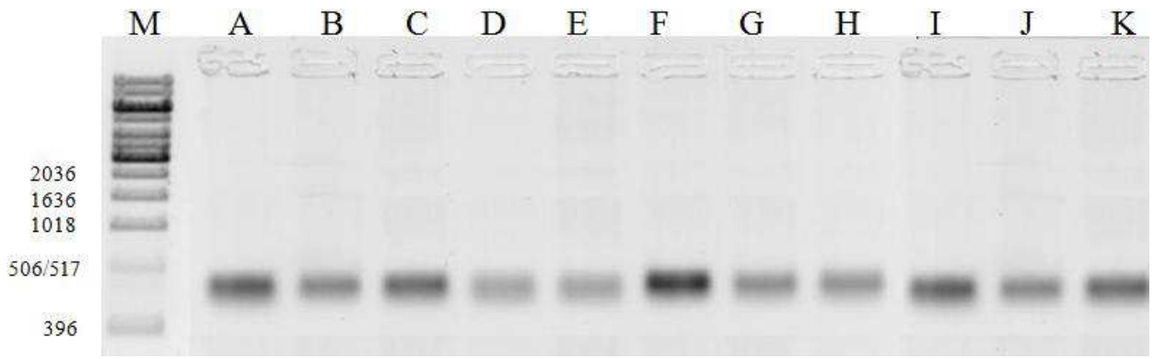

Fig. 1. Nuclear region amplifications that includes the spacers EF11-EF22. Cultivars and interspecific hybrids: A- *H. psittacorum* cv. Sassy; B- *H. psittacorum* cv. Red Gold; C- *H. psittacorum* x *H. spathocircinata* cv. Golden Torch Adrian; D- *H. psittacorum* cv. Suriname Sassy; E- *H. psittacorum* x *H. spathocircinata* cv. Red Opal; F- *H.* x nickeriensis; G- *H. psittacorum* x *H. spathocircinata* cv. Golden Torch; H- *Heliconia* sp.; I- *H. psittacorum* cv. Lady Di; J- *H. psittacorum* cv. Strawberries e Cream; K- *H. psittacorum* cv. St. Vincent Red. (M = 1 kb DNA ladder).

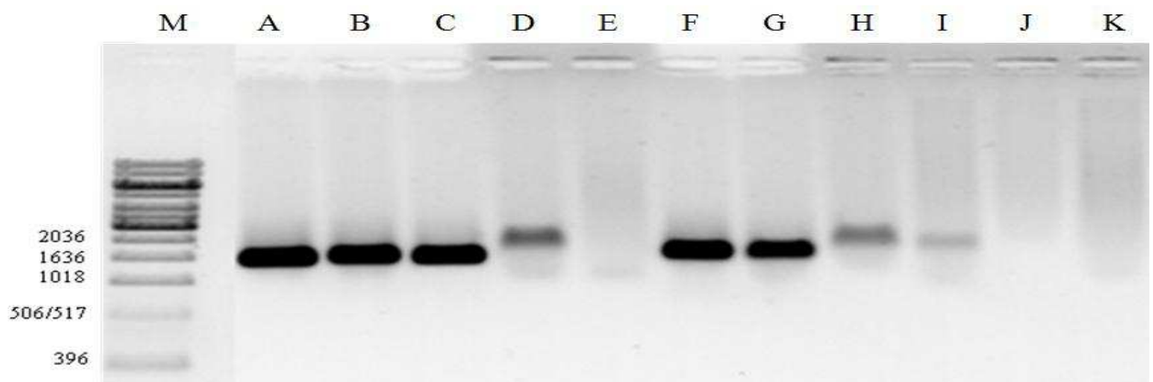

Fig. 2. Chloroplast regions with *primers trnL-trnF* amplifications. Cultivars and interspecific hybrids: A- *H. psittacorum* cv. Sassy; B- *H. psittacorum* cv. Red Gold; C- *H. psittacorum* x *H. spathocircinata* cv. Golden Torch Adrian; D- *H. psittacorum* cv. Suriname Sassy; E- *H. psittacorum* x *H. spathocircinata* cv. Red Opal; F- *H.* x nickeriensis; G- *H. psittacorum* x *H. spathocircinata* cv. Golden Torch; H- *Heliconia* sp.; I- *H. psittacorum* cv. Lady Di; J- *H. psittacorum* cv. Strawberries e Cream; K- *H. psittacorum* cv. St. Vincent Red. (M = 1 kb DNA ladder).

3.2 Internal transcribed spacers

From the data generated by ITS markers and the analysis of the dendrogram (Fig. 3), it was observed the formation of two main groups (GI and GII) well sustained. The GI group, is constituted by *Heliconia* sp., while, the other, more representative, GII, is subdivided into two other subgroups, SG A and SG B.

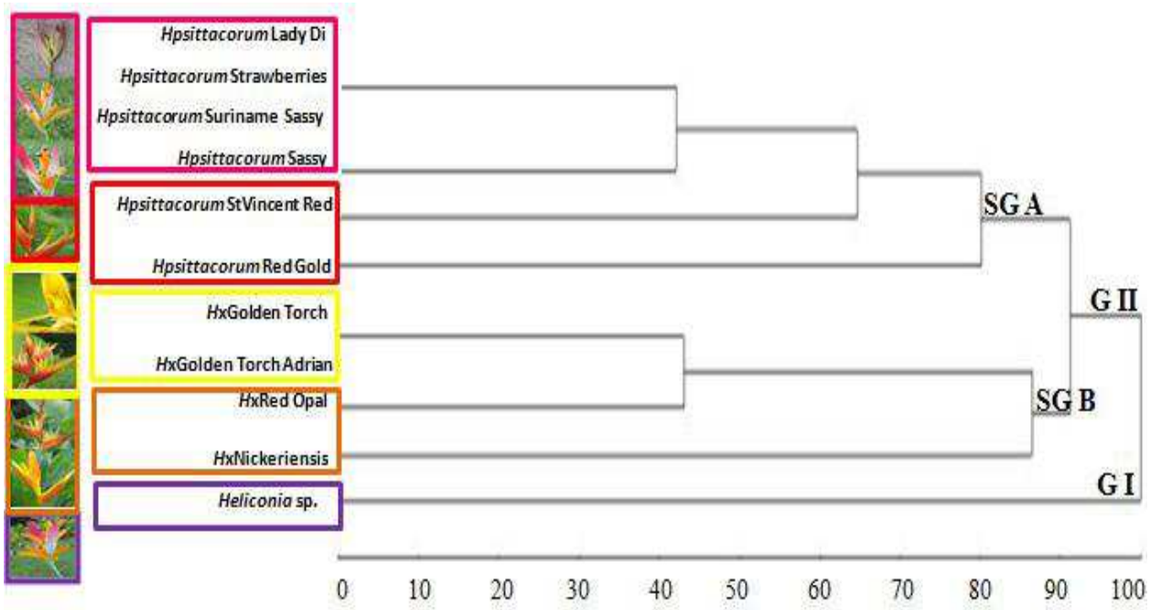

Fig. 3. Cluster analysis on 11 genotypes of the UFRPE Heliconia Germplasm Collection, used in this study, through ITS. G I= Group I; G II= Group II; SG A= Subgroup A; SG B= Subgroup B.

The first group GI, consisting of *Heliconia* sp., that according to farmers, is identified as *H. psittacorum* cv. Sassy and was more divergent from the other genotypes. The hypothesis that it is a new cultivar is supported by the fact that it was the only one that came from the state of Alagoas. It presents floral features intermediate between the triploid cultivars (Costa *et al*., 2008) of the subgroup SG A, bracts of pink and lilac, which presents individual characteristics such as, ovarian (OV) green distally and yellow green proximally, pedicel (PD) yellow green and sepals (SE) orange with indistinct blackish green area distally.

In the second group GII, subgroup SG A, formed by triploid cultivars of *H. psittacorum*: cvs. Suriname Sassy and Sassy, that present bracts with pink and lilac, was also included genotypes from the state of São Paulo, *H. psittacorum* cv. St Vincent Red and *H. psittacorum* cv. Lady Di.

The subgroup SG B was formed by the hybrid *H. psittacorum* x *H. spathocircinata* cvs. Golden Torch, Golden Torch Adrian and Red Opal, with bracts yellow and red. In this subgroup on an external position, it was observed *H.* x *nickeriensis*, that is supposed to be an hybrid between *H. psittacorum* x *H. marginata*.

The hybrids showed low levels of similarity, around 12% of these comparisons reached levels above 50% (Table 3), probably because they are the result from supposed crosses between genetically distant parents or even the influences of epigenetic factors.

Genotypes	*Hpsittacorum* Sassy	*Hpsittacorum Red Gold*	*Hx GoldenTorch Adrian*	*Hpsittacorum* Suriname Sassy	*Hx* Red Opal	*Hx* Nickeriensis	*Hx GoldenTorch*	*Heliconia* sp.	*Hpsittacorum* Lady Di	*Hpsittacorum* Strawberries	*Hpsittacorum* StVincent Red
Hpsittacorum Sassy	1.00										
Hpsittacorum Red Gold	0.38	1.00									
Hx GoldenTorch Adrian	0.58	0.36	1.00								
Hpsittacorum Suriname Sassy	0.45	0.33	0.44	1.00							
Hx Red Opal	0.18	0.28	0.25	0.40	1.00						
Hx Nickeriensis	0.41	0.44	0.55	0.37	0.33	1.00					
Hx GoldenTorch	0.18	0.28	0.25	0.40	1.00	0.33	1.00				
Heliconia sp.	0.45	0.50	0.44	0.42	0.16	0.37	0.16	1.00			
Hpsittacorum Lady Di	0.09	0.14	0.12	0.13	0.10	0.16	0.12	0.21	1.00		
Hpsittacorum Strawberries	0.09	0.14	0.12	0.13	0.10	0.16	0.12	0.21	0.10	1.00	
Hpsittacorum StVincent Red	0.09	0.13	0.10	0.13	0.10	0.16	0.12	0.20	0.10	0.10	1.00

Table 3. Genetic similarity between 11 cultivars of *H. psittacorum* and interspecific hybrids of the UFRPE Heliconia Germplasm Collection used in this study

4. Discussion

It was not possible to obtain DNA with acceptable quality from *Heliconia* using the conventional methodology, as mentioned by Kumar *et al*. (1998), in an earlier study with molecular markers in heliconia.

4.1 Primers selection

Band patterns variation may be related to high occurrence rate of base substitution and the great possibility of indels accumulation (events of inserts and/or deletions of nucleotides), moreover, these sequences are difficult to identify (Albert *et al*., 2002). The study with a great number of genotypes aims to explain the inheritance of the chloroplast, which may vary according to the subgenus and be useful for genetic diversity studies of the group. These *primers* (*trnL-F*) have been successfully used in genetic diversity analysis of Orchidaceae (Kocyan *et al*., 2004) and Bromeliaceae groups (Sousa *et al*., 2007).

4.2 Internal transcribed spacers

In the absence of more precise evidence, it was decided to keep the genotype, here called *Heliconia* sp., as a specie not yet identified. It is assumed as a new cultivar of *H. psittacorum* cv. Sassy that occurred due to different geographic conditions. In fact, this finding requires further studies. Other molecular markers can be used to solve this issue, as did Kumar *et al*. (1998), that using RAPD, found that two triploid cultivars, Iris and Petra were the same genotype. Sheela *et al*. (2006) by using RAPD, found that cvs. St Vincent Red and Lady Di, were also grouped in the same subgroup. Thus, assuming that these genotypes formed a subgroup brother of triploid cultivars *H. psittacorum* cv. Sassy and cv. Suriname Sassy, presenting 2n = 36 (Costa *et al*., 2008), leads to the assumption that cvs. St Vincent Red and Lady Di are supposed to be triploid, corroborating with the similar banding pattern among these four genotypes in primer combination ITS3-ITS4.

The group that gathered the hybrids *H. psittacorum* x *H. spathocircinata* cvs. Golden Torch, Golden Torch Adrian and Red Opal was expected, once the nrDNA has biparental inheritance, and it is a nuclear molecular marker. *H.* x *nickeriensis* belongs to the *Heliconia* subgenus and *Pendulae* section (Kress *et al*., 1993), this subdivision is based on the consistency of vegetative structure, and staminodes and style shape, especially in the pending heliconia. *H. marginata*, alleged parent, has pending inflorescence, and yet, differ from other hybrids that are crosses between *H. psittacorum* x *H. spathocircinata* and belongs to the *Stenochlamys* subgenus and *Stenochlamys* section (Kress *et al*., 1993). Using RAPD markers to study genetic variability and relationship between 124 genotypes of the genus *Heliconia*, Marouelli *et al*. (2010), managed to gather interspecific hybrids of *H. psittacorum* in the same clade.

The hybrids showed small similarity that can be explained by the coevolution hypothesis, which considers the great genetic diversity of the genotype in the center of origin, once in northeast Brazil is frequently encountered native populations of *H. psittacorum*. Moreover, there is a wide variety of *H. psittacorum* hybrids described in literature, especially *H. spathorcircinata*, confirming the potential of this specie to form hybrids (Berry and Kress, 1991).

The influence of epigenetic factors in the phenotype of an organism and therefore in obtaining hybrids of *Heliconia* should be an issue to be raised. Characteristics of the transmissibility of an individual to other generations are not only linked to genes, the cell should be considered with its cytoplasm, mitochondria and genetic material carried in its structure, as well as the organism as a whole, and the complexity of the environment (Pearson, 2006). Another factor to be considered is the cytosine methylation of the genetic material, also responsible for gene silencing, causing changes in the phenotype, and according to most recent works can be passed to subsequent generations, thus causing greate genetic diversity among individuals of the same specie.

Routinely, new *Heliconia* species have been described and others have been included as synonyms on each revision of the genus or subgenus; but, there is still controversy among authors. This situation suggests the need for a careful review of this group, since the visual botanical identification, may lead to imprecise denomination for the species that are being cultivated.

Although some diversity studies about the Heliconiaceae family have been undertaken in recent years, its classification remains opened, therefore, new genetic markers for the group are required to elucidate these classification issues. The results revealed that there was no repetition of genetic material among the cultivars and interspecific hybrids of *H. psittacorum* evaluated, indicating the necessity to use other regions that could provide potentially informative characters. In conclusion, the genetic diversity nuclear and chloroplast DNA regions observed to study in *Heliconia psittacorum* cultivars and interspecific hybrids, are information promising to be taken in account as a first step towards genetic improvement.

5. Acknowledgements

The authors thank the National Council of Scientific and Technological Development (CNPq) and the Coordination for the Improvement of Higher Education (CAPES) for the scholarship of the first author, the BNB for the financial support, the Bem-Te-Vi Farm, the RECIFLORA association, researcher scientist Dr. Carlos E. F. de Castro Campinas Agronomic Institute (IAC) and trainees of the UFRPE Floriculture Laboratory.

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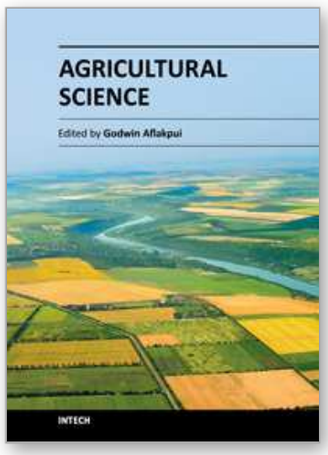

Agricultural Science
Edited by Dr. Godwin Aflakpui

ISBN 978-953-51-0567-1
Hard cover, 252 pages
Publisher InTech
Published online 27, April, 2012
Published in print edition April, 2012

This book covers key areas in agricultural science, namely crop improvement, production, response to water, nutrients, and temperature, crop protection, agriculture and human health, and animal nutrition. The contributions by the authors include manipulation of the variables and genetic resources of inheritance of quantitative genes, crop rotation, soil water and nitrogen, and effect of temperature on flowering. The rest are protecting crops against insect pests and diseases, linking agriculture landscape to recreation by humans, and small ruminant nutrition. This book is a valuable addition to the existing knowledge and is especially intended for university students and all professionals in the field of agriculture.

How to reference
In order to correctly reference this scholarly work, feel free to copy and paste the following:

Walma Nogueira Ramos Guimarães, Gabriela de Morais Guerra Ferraz, Luiza Suely Semen Martins, Luciane Vilela Resende, Helio Almeida Burity and Vivian Loges (2012). Genetic Diversity Analysis of Heliconia psittacorum Cultivars and Interspecific Hybrids Using Nuclear and Chloroplast DNA Regions, Agricultural Science, Dr. Godwin Aflakpui (Ed.), ISBN: 978-953-51-0567-1, InTech, Available from: http://www.intechopen.com/books/agricultural-science/genetic-diversity-analysis-of-heliconia-psittacorum-cultivars-and-interspecific-hybrids-using-nuclea

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51000 Rijeka, Croatia
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InTech China
Unit 405, Office Block, Hotel Equatorial Shanghai
No.65, Yan An Road (West), Shanghai, 200040, China
中国上海市延安西路65号上海国际贵都大饭店办公楼405单元
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