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**Faculty of Tropical AgriSciences**

# Morphological and molecular characterization of 11 varieties of native chilli peppers (*Capsicum* spp.) of the Peruvian Amazon

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## Statutory declaration

I hereby certify that I have elaborated my thesis independently, only with the expert guidance of my thesis tutor Doc. Dr. Ing. Eloy Fernandez and Ing. PaeDr. Jana Žiarovská, Ph.D. from Slovak University of Agriculture in Nitra.

I further declare that all data and information I have used in my thesis are stated in the reference.

In Prague April 2013 …….

Bc. Goldis PERRY DAVILA

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## Abstract (EN)

Peruvian Amazonia is one of the most diverse ecosystem of the world. With its specific location and climatic conditions forms a habitat for more than 50% species and represents origin of many plant species with high economic, cultural and medicinal importance. Among these species are native chilli peppers (*Capsicum* sp.). Till today there isn't complete information about diversity of Amazonian chilli peppers although its importance is increasing. For present research were chosen and collected 11 native chilli peppers with the main purpose to measure their genetical distance. First the samples were described morphologically according to International standardized descriptors for *Capsicum*. Biological material, especially seeds, was first homogenized and then tested in laboratory using PCR and modern IPBS method to provide genetical description. Despite the fact that the samples were diverse, the 11 varieties of analyzed chillies belong to 3 species: *C. chinense*, *C. baccatum* and *C. frutescens*. The lowest dissimilarity rate was found in charapita amarillo and charapita rojo chilli (0,38) and in the other hand the highest rate was recorded in chilli Pinchito de mono (0,73). At least we compare the results to practice finding possible substitute to widely used charapita amarillo, which has high local economic and cultural importance. The chilli trompito amarillo presents similar characteristics and promising good yield as well. This research forms a good data base for further investigation and studies.

**Key words:** Amazonian chilli peppers, IPBS (Inter Primer Binding Site), morphologic descriptors, PCR, genetic distance

## Abstract (ES)

La Amazonia peruana es uno de los mas diversios ecosistemas en el mundo. Con su localizacion y clima especifico forma un habitat para mas de 50% de especies descritas y representa el lugar de origen de muchas especies de plantas con gran importancia economica, cultural y medicinal. Entre estas especies se encuentran los ajies nativos (*Capsicum* sp.). Hasta ahora no existe una informacion completa sobe la diversidad de ajies amazonicos a pesar que su popularidad e importancia crece. En el presente estudio fueron seleccionadas y colectadas 11 variedades de ajies nativos de la amazonia peruana, con el objetivo principal de medir su distancia genética. Como primer paso, las muestras fueron descritas morfologicamnete de acuerdo a un descriptor internacional estandarizado para *Capsicum* sp. El material biologico, especialmente semillas, fueron homogenizadas y luego analyzadas en laboratorio empleando el metodo de PCR y el moderno metodo de IPBS que proveyo la descripcion genetica. A pesar que las muestras eran diversas, se concluyo de que las 11 variedades en estudio, pertenecen a 3 especies: *C. chinense, C. baccatum* y *C. frutescens*. La disimilaridad mas baja fue encontrada en las variedades aji charapita amarillo y charapita rojo (0,38) y en el valor mas alto fue en caso de la variedad pinchito de mono (0,73). finalmente se compararon los resultados de las caracteristicas de cada variedad buscando un sustituto posible para aji charapita amarillo con un amplio rango de usos y gran importancia economica y cultural. El aji trompito amarillo presenta las charactersisticas similares, prometiendo altos rendimientos. Esta investigación forma una buena base de datos para proximos investigaciones y estudios.

**Palabras claves:** ajíes amazónicos, IPBS (Inter Primer Binding Site), descriptores morpfológicos, PCR, distancia genetica

## Abstract (CZ)

Peruánská Amazonie je jedním z nejbohatších ekosystémů světa. Díky své unikátní poloze a klimatu tvoří životní prostředí pro více než 50% popsaných druhů a je místem původu mnoha rostlin s vysokou ekonomickou a kulturní a lékařskou důležitostí. Mezi těmito druhy jsou také amazonské papričky (*Capsicum* sp.). Dodnes neexistuje kompletní informace o diverzitě těchto papriček, přestože jejich popularita a důležitost stále roste. Pro tuto studii bylo vybráno a sebráno 11 původních papriček s hlavním cílem determinovat jejich genetickou vzdálenost. Vzorky byly nejprve morfologicky popsány dle mezinárodních standardizovaných deskriptorů pro rod *Capsicum*. Biologický materiál, zejména semena, byla homogenizována a poté analyzována v laboratoři s požitím PCR a moderní IPBS metody pro genetický popis. Přestože byl materiál velmi diverzní, vybraných 11 variet papriček patřilo ke 3 druhům: *C. chinense, C. baccatum* a *C. frutescens.* Nejmenší genetická vzdálenost byla zjištěna u papričky charapita amarillo a charapita rojo (0,38) and na druhé straně největší vzdálenost byla u papričky pinchito de mono (0,73). Nakonec jsme provedli srovnání výsledků s praxí ve snaze doporučit vhodný substitut k široce využívané varietě charapita amarillo s vysokou ekonomickou a kulturní důležitostí. Varieta trompitos amarillo vykazuje velmi podobnou charakteristiku a slibuje také vysoký výnos. Tento výzkum představuje dobrou databázi pro další výzkum a studie.

**Klíčová slova:** amazonské papričky, IPBS (Inter Primer Binding Site), morfologické deskriptory, PCR, genetická vzdálenost

# 1. Introduction

The Amazonian rainforest is the unique and one of the most diverse ecosystems in the World. With its diverse vegetation cover, water resources and specific microclimate forms a habitat for more than 50% of described plant and animal species. However, it is an amazing source of plants of numerous uses such as various edible plants, medicinal plant species, technical and ornamental plant, etc. Lot of plants well known locally, but not yet scientifically, are used by local native people for centuries and has even mythological importance. Those are, among others, the chilli peppers.

The first notes about chilli peppers in Peru were found during archeological researches in Tacna and Ancash provenience. The chilli peppers were "eternized" in 2500 b.c. on local ceramics used for religious purposes. Chilli peppers are also on historical textiles and sculptures. The famous Inca tribe was using chilli peppers to punish prisoners by concentrated smoke of burned dried chilli pepper pods. In historical battles they used chilli peppers as an alternative of today's defense pepper spray. Around 14$^{th}$ century the Peruvian chilli peppers from mountain regions was discovered by Spanish colonists and valuable condiment. Sounth America gives to the world not only chilli peppers but also highly important crops as maize (*Zea maiz*) and potato (*Solanum tuberosum*) on which nowadays depends nutrition of millions of people. But the chilies from Amazonian lowlands were still waiting for its discovering. First users of Amazonian picante "gold" were native indigenous tribes and become an important part of its diet and medicines.

Picante pods typical for Asiatic and Indian food, are known already for centuries, but till last decades, the chilli peppers of Amazonia weren't known world widely. Nowadays the research of edible plants is focused on new species and varieties of Amazonian chilli peppers which have excellent characteristics on field of food and medicine as well. Chilli peppers (*Capsicum* sp. of *Solanaceae* family) make naturally a number of varieties and for the exact description is a needed modern morphological and genetical descriptive method. Information about genetical species/variety identity provides a good basis for modern breeding method for better desired agriculture and dietetic characteristics. The fact that the Chilli has major economic importance to many countries explains the use of valuable scientific methods, as mentioned here above, not only for scientific reasons but also underlines its economic value for further investigations and yield optimization.

# 2. Literature review

## 2.1. Taxonomic description of *Capsicum* genus

Genus *Capsicum* is grouped in the Botanical Family called *Solanaceae*, which includes economically important crops such as tomato (*Lycopersicon esculentum* Mill), potato (*Solanum tuberosum*), tobacco (*Nicotine tabacum*) and eggplant (*Solanum melongena* L.), this genus was described by Carlos Linnaeus and published in 1753 in his monumental work, Species plantarum. It is believed that the name assigned is derived from Greek *kapto*, meaning "itching", that is its main feature (Salazar and Silva, 2004). The genus *Capsicum* has a wide genetic diversity, composed by 27 species, being five domesticated: *C. annuum, C. baccatum, C. frutescens, C. chinense, C. pubuscens* and 22 semi-domesticated and wild ones: *C. buforum, C. campylopodium, C. cardenasii, C. chacoense, C. coccineum, C. cornutum, C. dimorphum, C. dusenii, C. eximium, C. glapagoensis, C. geminifolium, C. hookerianum, C. lanceolatum, C. leptopodum, C. minutiflorum, C. mirabile, C. parvifolium, C. praetermissum, C.scolnikianum ,C. schottianum, C. tovarii, C. villosum* (Reifschneider, 2000; Kumar Basu and Krishna De. 2003), other authors affirm that the number of species comprising the genus *Capsicum* is 20 to 23 and which taxonomy of the genus is confusing, and sometimes it is difficult to identify an accession using only subjective morpho-agronomic data (Moscone *et al*,. 1993 and Pickersgill, 1997).

*Capsicum* botanical classification has been difficult due to the high number of varieties, the lack of defined characteristics and the absence of barriers for hybridizing marked species for at that the criteria have varied (Pérez Castañeda, 2010). According to Bosland and Votava (1999) the genus *Capsicum* is into the following taxonomy:

Kingdom: *Plantae*
Division: *Magnoliophyta*
Class: *Magnoliopsida*
Order: *Solanales*
Family: *Solanaceae*
Genus: *Capsicum*

## 2.2. Botanical description

The significant diagnostic characteristic of *Capsicum* is an annual or perennial glabrous herb or undershrub which has an erect, branched growth habit (Figure 1.).

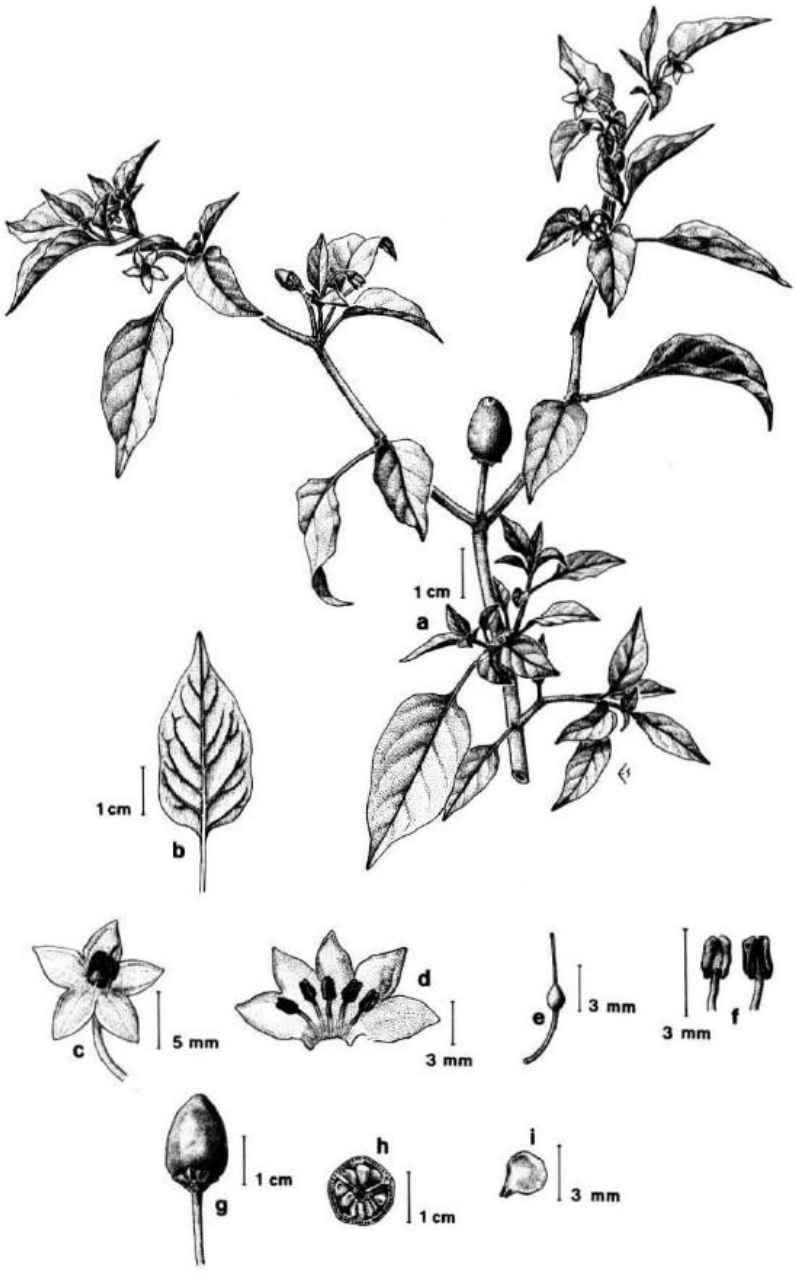


Figure 1. Capsicum annuum var. annuum.
A- branch with leafs, flowers and fruits, B – leafs, C – flower, D – open corolla with stamens, E – pistil on pedicel, F – stamen, G – fruit, H – transversal profile of the fruit, I – seed (Nee, 1986).

Plants grow to a height of 60–75 cm, with a large number of fruit produced on lateral branches. New branches are produced at every flower node. Leaves that alternate, are entire or repand. Flowers are pedicelled, axillary, solitary or two-three together. Sepals are connate in a subentire or minutely five-toothed calyx and much shorter than the fruit. Petals are five, connate in a rotate corolla. The lobes are valvate in bud and the five stamens adnate nearly to base of corolla-tube. Filaments are short and the anthers don't exceed the filaments. Fruit is globose or elongated or irregularly shaped, many seeded berry. Seeds are discoid, smooth or subscabrous. It comprises a very wide range of fruit forms according to the species, varying in shape, color, pungency and position of fruits. (D'arcy and Eshbaugh, 1974; Kumar Basu and Krishna De, 2003). The chilli pepper plants have hermaphrodite flowers and reproductive system type autofertilization. However, the levels of cross pollination vary between and within species. Studies have shown that cross-pollination can occur in a range of 2 to 90% (Tanksley, 1984), which allows placing them in group intermediary between allogamous and autogamous. In domesticated species, the stigma is at the same level anthers increasing the possibility of selfing, whereas in wild stigma is above the anthers facilitating outcrossing (Casali and Couto, 1984).

## 2.3. Origin and distribution

The original distribution of *Capsicum* appears to have been from the south of Mexico, extending into Columbia, however some archaeological studies published by Dillehay (2012) about seeds of *Capsicum* recovered during excavations from various temporal phases at the Preceramic sites of Huaca Prieta and Paredones (7500-4000 BC) in north coastal Peru affirm that *Capsicum* has its origin in South America, this in comparison archaeological records in Mexico (6500-5500 BC). (Nuez-Viñals *et al*., 1998; Kumar Basu and Krishna De, 2003 and Moreira *et al*, 2006). The general consensus among botanists is that the nuclear origin area for the *Capsicum* genus is in highland Bolivia on the eastern slopes which is also the purported origin of the domesticated *C. pubescens*; from there, the wild *Capsicum* species radiated outwards through the Americas due to dispersal by birds and possibly humans (Esbaugh, *et al*., 1983 and Pickersgill 1977, 1988, 2009). Other principal *Capsicum* species as *C. baccatum* is thought to have been domesticated in lowland coastal Peru, while *C. chinense* and *C. frutescens* may have more tropical roots in the northeastern Amazon (Moses and Umaharan, 2012; Pickersgill, 1972; Aguilar-Meléndez, 2006, 2009; Hernández-Verdugo, 2001, Perry and

Flannery, 2007). *C. annuum*, on the other hand, was domesticated in Mexico (Pickersgill, 1972 and Aguilar-Melendez, 2006).

In the fifteenth century, the plant was introduced into Spain by Columbus and into India by the Portuguese, from where it spread widely. Subsequently, the prolonged viability, easy germination and easy transportation assisted its spread all across the globe. Chilli is essentially a crop of the tropics and grows better in hotter regions. It is cultivated over large areas in all Asian countries, Africa, South and Central America, parts of USA and southern Europe, both under tropical and subtropical conditions. The species *C. annuum* var. annuum, *C. chinense*, *C. pubescens*, C. baccatum var pendulum, and *C. frutescens* are the main cultivated (Loaiza-Figueroa *et al*., 1989; Hernández *et al*.1998 and Morán *et al*., 2004).

In the Amazon the species of the genus *Capsicum* are one important source of income for the local agricultural population (indians and non-indians), due to the fact that this region is an important center of diversity of the genus *Capsicum*, particularly of the species *Capsicum chinense*, (Silva Filho *et al*. 2001 and Costa *et al*., 2009).

## 2.4. Ecology

The majority of the *Capsicum* species grow in Asian, Central and Latin American countries are pungent, while in Europe countries cultivation of less pungent or non-pungent peppers are more common. Hot chillies, which are usually grown for red dry chillies, are raised as a rainfed crop in several parts of the world that receive an annual rainfall of around 80-100 cm, hot peppers have more water requirements than non-hot types (Valenzuela, 2011). The optimal growth conditions for the development of *Capsicum* ranging from 7-29 ° C temperature, annual precipitation of 0.3 to 4.6 m soil pH of 4.3 to 8.7. *Capsicum* species are sensitive to cold and generally grow best in well drained soils, sandy or loamy black soil. Plantations are established by seed or transplant (Bosland, 1996).

Chillies are shallow rooted crops and are very sensitive to soil moisture variations, more than half of the root system is concentrated in the 5-1 5 cm layer. Chilli plants cannot withstand water stagnation and excessive moisture at any of the growth stages. If saturated conditions continue for more than 24 hours, the plants may be killed. Dew and heavy rain at flowering

are injurious to the crop, causing flower buds and young fruits to drop off (Kumar Basu and Krishna De, 2003)

## 2.5. Uses

The chilli is a worldwide popularity spice of appreciated for its color attributes, chilli peppers are consumed fresh or in a variety of processed products in many cuisines worldwide, they are used as condiments or spices to add flavor or pungency to dishes. In recent years, chilli varieties being utilized for a wide range of food processing, medicine development, pest an animal control and even in law enforcement (Kokoška, 2003 and Kumar, *et al*., 2003). In the salsa sales now surpass ketchup sales, reflecting on the popularity of Mexican dishes. Chilli peppers are used medicinally in Latin America and Africa. In many countries, chillies are part of the daily diet. Some cultivars are also used as ornamentals. In many regions where chilli peppers are widely consumed, they represent one of the few, if not the only, vegetable added to the diet to provide flavor, spice, and variety to grain or root-crop-based diets. The chilli Pepper is increasingly recognized as an excellent source of health-related metabolites, such as ascorbic acid (vitamin C), carotenoids (provitamin A), tocopherols (vitamin E), flavonoids and capsaicinoids (Howard and Wildman 2007). The strong pungency and spicy flavor of the chilli peppers, has been attributed to a family of compounds called "capsaicinoids", always present and only present, in the fruits of the *Capsicum* genus varieties in different amount, varying significantly from one to another. These are interesting compounds that beyond their roles as flavor ingredients have also medical, toxicological and therapeutic implications, in addition to their widespread use as a neuro-pharmacological tool, due their effective action in the treatment of painful medical conditions. Indeed, capsaicinoids have been reported as having antioxidant effect and antibacterial action on certain groups of bacteria (Pino *et al*., 2007; Kurian and Starks, 2002). Processed chilli peppers are found in a variety of products including main dishes, meats, salad dressings, dairy products, beverages, candies, baked products, snack foods, salsas, hot sauces, and even in ice cream. Extracts are also used in pharmaceuticals, as medicinals, and in cosmetic products (Bosland and Votava, 2000).

## 2.6 Characterization studies in *Capsicum*

Nowadays, there are a high number of publications that detail the essential characteristics of cultivated and wild species of the genus *Capsicum*, because the morphologic characterization

and the evaluation of the genetic diversity are essential to maintain an active basis for the exploration of the genetic variability in breeding programs (Viana *et al*. 2006; Arriel *et al*., 2007 and Lannes *et al*., 2007). Studies made by Ibisa (2012), in the taxonomy and genetic diversity of domesticated *Capsicum* species in the Andean region describe the characteristics using molecular markers and morphological descriptors of *Capsicum* species found in the Andean region looking for useful plants for breeding new cultivars or hybrids, other studies made in Mexico and Brazil focuses on determining the diversity within the complex *Capsicum annuum-chinense-frutescens* and *Capsicum annuum-chinense-frutescens*, mention the importance of the morphological and molecular characterization for understand this complex among species (Walsh and Hoot, 2001; Barrios *et al*., 2007; Jarret and Berke, 2008 and Büttow *et al*., 2010). For chilli a comparison and characterization of the plant phenotype is the simplest approach for the detection of genotypes and the assessment of genetic diversity; however, phenotypic evaluation is influenced by environment and might not distinguish between closely related genotypes (Rodriguez *et al*., 1999).

Chilli peppers have been object of study mainly due to containing capsaicin, because is the most abundant of the capsaicinoids present in the chilli and mainly responsible for pungency of it. (Oboh *et al*., 2007), as well as carotenoids and phenolic compounds, which are used as natural pigments and antioxidant agents (Suna, *et al*., 2008). The taxonomy of the genus *Capicum* is confusing, and sometimes it is difficult to identify and to characterize an accession using only subjective morpho-agronomic data. Markers of morphological characteristic for *Capsicum* especially that focus in flower and fruit morphologies have been known for very long time and these visually observed markers are small in number and might have epistatic effects (Rodriguez *et al*., 1999). However, DNA markers such as RFLP, RAPD, AFLP, and micro-satellites have been beneficial by being large in number and not affected by the environment (Dominguez Barradas, 2001 and Da Costa *et al.*, 2006).

## 2.7. General list of native chilli peppers produced in Peruvian Amazon – Ucayali

Cerecito rojo (Figure 2/6) is the local name of relatively small chilli pepper fruit of deep red color with a specific picante taste. It grows on chilli plants of medium height. The estimated yield is 5,6 t/ha. Local people relates it to other chilli pepper called Cerecito Amarillo (Figure 2/7) which has a similar form and growth and differs with the yellow fruit color. This fruit is a little bit less picante which refers also to lower content of capsaicin.

Another group of often related chilli peppers are Charapitas. Local inhabitants of Ucayali region sort them according to the size and color. All of them have an important role on local markets where are highly required. The people prefer mostly the Charapita Amarillo (Figure 2/3.) for its special taste and multifunctional use in picante sauces. This fruit is one of the smallest chilli peppers, it weighs about 3 grams. It yields per hectare reaches up to 12 tones. When it is not accessible on the market, the people substitute it easily with Charapón Amarillo (Figure 2) whose fruit is double than Charapita amarillo.The red forms of those chilli peppers: Charapita rojo (Figure 2/1) and Charapón rojo (Figure 2) contain only about 150 mg of capsaicin per 100 gr, that why it is not so required on local markets for its gentle taste. Its production reaches about 6 up to 8 tons per hectare.

The Rocotito (Figure 2) has relatively big fruits, up to 12 grams. It got its name according to the similarity with Rocoto chilli (*Capsicum pubescens*.) which contains high levels of capsaicin and is used mainly in mountain regions of Peru. Rocotito presents a smaller version of Rocoto chilli pepper. Trompito Amarillo (Figure 2/5) and Trompito rojo (Figure 2/4) are triangulate chilli peppers about 3 grams in weight and quite popular for its shape. They are produced in estimated amounts of 15 tons per hectare and are sold pickled in other species in local, national and international markets. The Chintito chilli pepper (Figure 2) is one of the smallest chillies growing in Amazonia. It is produced mainly for self-consumption for local farmers. In local markers is not so required for its small size. In the other hand the Ayuyo (Figure 2/2) has a stable position on local and national markets because it is a principal ingredient for national dishes. It is preferred for its purple color at premature stage and that's why, there are seasonally produced up to 13 tons per hectare. One of the less picante Amazonian chilli pepper is Aji dulce (Figure 2), which contains only 25 mg of capsaicin per 100 g. It is used in salads and sauces. The production of this gentle chilli reaches up to 25 tons per hectare. The Upia ucho (Figure 2/10) chilli is produced mainly for self-consumptions in rural zones (3 t/ha).

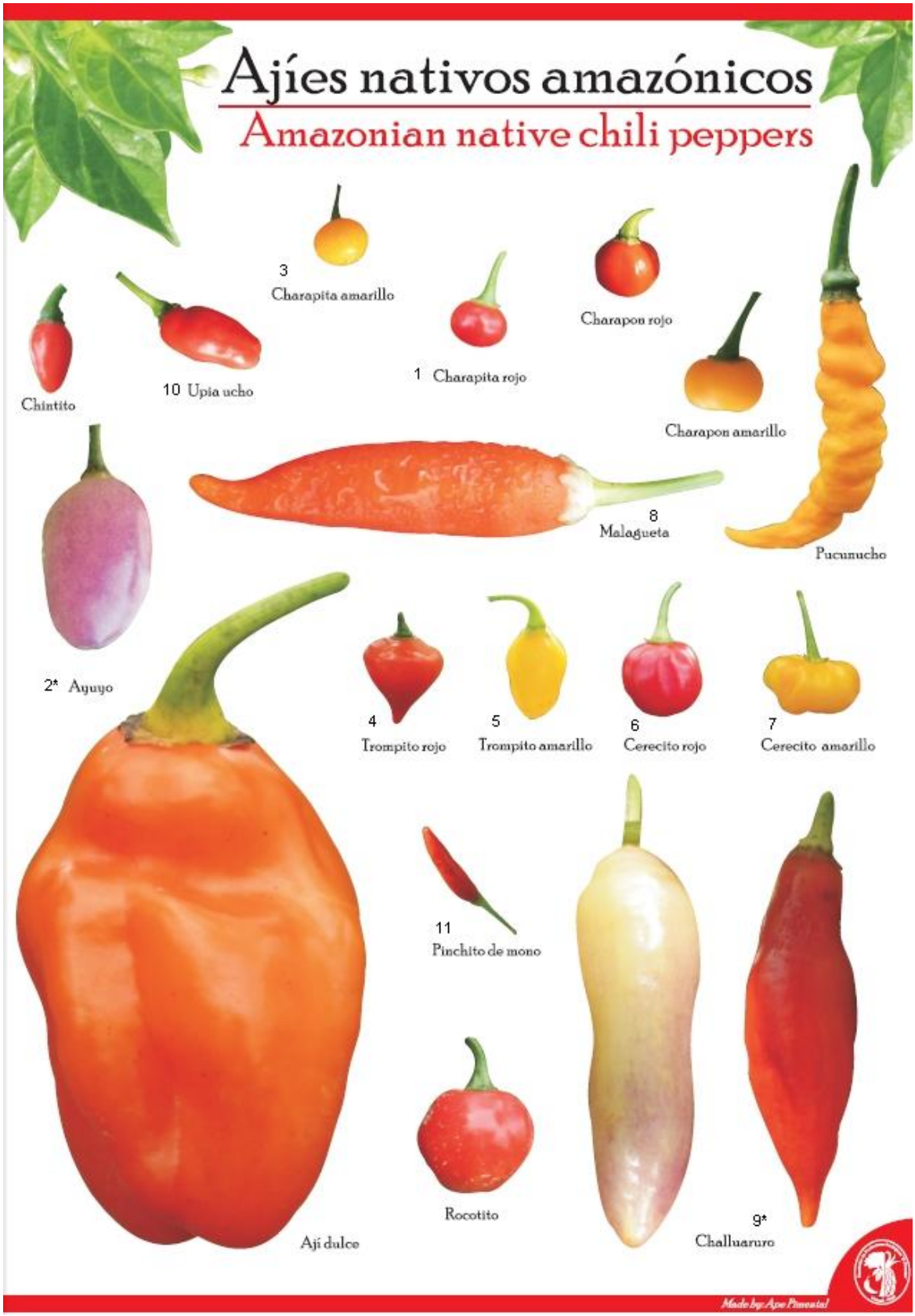


Figure 2. General list of Amazonian native chilli peppers.
*Only chillies used for present research are numbered. More photos are added in Appendix IV.*
** Purple color is typical for premature stage, mature fruit is red.*

Among elongated shaped chilli peppers we can found Malagueta (Figure 2/8) chilli, with quite small fruits about 1,5 g of weight with high content of capsaicin. It is used sporadically and produced mainly for pickleds. The Challuaruro (Figure 2/9) is known for its purple color in premature stage. It is used, as the previous one, for pickleds sold in national market and it's produced in amounts of 6 t/ha. The moon-shaped chilli is Pucunucho (Figure 2). For its wrinkled surface is used as decoration in many houses, it is sold dried or pickled or in sauces. Its production reaches 4 t/ha. Popularity of this chilli is mainly for its appearance, because its taste is one of the strongest in the amazon. The last chilli pepper listed here is the Pinchito de mono (Figure 2/11) also called "Pipí". With its 0,8g of weight is definitely the smallest one in this list, but has one of the highest yields, up to 20 t/ha. It is required on national and international markets, where is added to decoration pickleds, to spicy sauces and including in new chocolates. It has high content of capsaicin which is underlined by its strong taste.

According to APEGA (2009) till now there is not known deeply the real diversity of Peruvian native chilli peppers and needs modern methods to investigate it.

# 3. Modern characterization processes on morphological and genetical basis

## 3.1. IPGRI Morphological descriptors: a worldwide standardized description method

The Bioversity International (International Plant Genetic Resources Institute - IPGRI) is an independent international scientific organization that seeks to advance the conservation and use of plant genetic diversity for the well-being of present and future generations. It is one of 16 Future Harvest Centers supported by the Consultative Group on International Agricultural Research (CGIAR), an association of public and private members who support efforts to mobilize cutting-edge science to reduce hunger and poverty, improve human nutrition and health, and protect the environment (de Vicente and Fulton, 2003).

According to IPGRI (1995) the descriptor provides an international description format and thereby produces a universally understood 'language' for plant genetic resources data.

If the same scheme of descriptor data encoding is adopted world widely, then produce a rapid, reliable way of description and communication and will with the utilization of germplasm and as the background for molecular analysis. Bioversity International has developed three main types of standards: Crop descriptors, Multi-crop passport descriptors and Descriptors for genetic marker technologies.

Crop specific descriptors: It is an important tool in standardizing documentation systems, providing as it does an international format and a universally understood 'language' for plant genetic resources data (Bioversity International, 2007).

Multi-crop passport descriptors: The MCPD list is a reference tool that provides international standards to facilitate germplasm passport information exchange across crops (Bioversity International, 2007 and IPGRI, 1995).

Descriptors for genetic marker technologies: This list of descriptors defines a minimum set of data needed to describe accessions using biochemical and molecular markers, and defines community standards for documenting information about genetic markers. The document, which was originally based on some of the descriptors listed in the traditional evaluation category of the crop descriptors, is targeted at researchers using genetic marker technologies, to facilitate the generation and exchange of standardized genetic marker data. It also provides

descriptions of content and coding schemes that can assist in computerized data exchange (Bioversity International, 2007).

Morphological descriptors provide two main types of data as follows:

**Qualitative data** includes type of biological material (e.g. seeds, fruit, pollen, etc.), color of leafs, seed coat texture, flower color and its intensity. If the level of detail can be open, to interpretation by different users and can complicate future statistical analysis, it is recommended to carefully select the most representative states or include color chart codes, reference standards or drawings (Bioversity International, 2007). The same publication also recommends to use the Royal Horticultural Society (RHS) Color Charts for color descriptors which is strongly recommended for all ungraded color characters (the precise chart should be specified in the section where it is used).

**Quantitative data** consist of measures or counts that use numerical values, allowing statistical analyses, for which descriptions such as means and standard deviations are meaningful. Quantitative descriptors are those in which the expression covers the full range of variation from one extreme to the other. Different states of expression of quantitative data can be recorded using *discrete* (countable data, such as "number of plants"), or *continuous* (measurable data, such as plant height, weight, length) scales (Bioversity International, 2007).

According to the literature (IPGRI, 1995) there are various highly discriminating descriptors on the fruit basis (for detail see methodology).

## 3.2. Brief characterization of PCR and IPBS methods

According to Bartlett and Stirling (2003) the Polymerase chain reaction (PCR) method was developed and firstly used in 1983 by Kary Mullis, which was then awarded for the Nobel Prize in chemistry. Nowadays it is very common technique used in all biological and in medical research as well and has lot of uses (e.g. functional gene analysis, hereditary diseases analysis, genetic fingerprints, paternity etc.).

PCR method is based on thermal cycling (repeating of cycles of heating and cooling of the reaction) for DNA melting and enzymatic DNA replication. According to Bartlett and Stirling (2003) the primers (short DNA fragments) and DNA polymerase are the key components to

enable selective and repeated amplification. In the process of PCR the DNA is exponentially amplified in a chain reaction.

PCR reaction takes place in various steps as follows:

- Initialization step, when the reaction is kept up to 9 minutes under temperature of 94-96°C to activate DNA polymerase.
- Denaturation step where is realized the disruption of hydrogen bonds between complementary bases with the result of single-stranded DNA molecules.
- Annealing step: The reaction temperature is lowered to 50–65°C for 20–40 seconds allowing annealing of the primers to the single-stranded DNA template.
- Extension/elongation step: The temperature at this step depends on the DNA polymerase used. At this step the DNA polymerase synthesizes a new DNA strand complementary to the DNA template strand by adding dNTPs that are complementary to the template in 5' to 3' direction
- Final elongation: This single step is occasionally performed at a temperature of 70–74°C for 5–15 minutes after the last PCR cycle to ensure that any remaining single-stranded DNA is fully extended.
- Final hold: This step at 4–15°C for an indefinite time may be employed for short-term storage of the reaction.

Polymerase chain reaction is a complicated process which can fall for various reasons. That's why the protocols for PCR condition optimization were developed to minimize losses. The optimization is based on specified temperature, prevention of DNA contamination and purification of PCR products, addiction of polymerase enzymes can help with amplification of long or otherwise problematic regions of DNA, etc

Nowadays, an IPBS method had appear in genetics journals. According to Radová (2012) in recent genetics research, there was described a retrotransposon-based(elements found in *Eucaryota*) molecular marker technique, which is used to identify the plant varieties (Mondini *et al.*, 2009). Nowadays exist various retrotransposon-based molecular methods, which facilitate many researches, but lot of them require known sequence to design specific primers (Kalendar, 2011). Modern research is now focused on newly developed I-PBS

method which means "Inter-Primer Binding Site"). Radová (2012) states that I-PBS primers were designed according the highly conservative sequences called Primer Binding Site (PBS) to which tRNA binds, acting as a primer for reverse transcriptase during the replication cycle of retroviruses and LTR retrotransposons. I-PBS is a universal and efficient method from identification of polymorphism (Kalendar *et al*, 2010; Kalendar, 2011). The same author also describes that the IPBS amplification technique has proved to be a powerful DNA fingerprinting technology. This method allows genetic research of less known chillies and determines its genetic relation.

# 4 Objectives

The basic objective of this study is to evaluate the genetic distance among chosen11 varieties of native chilli peppers. For that purpose has to be realized several steps starting with the collection of biological material and morphological data using standardized international descriptors for *Capsicum* sp. and statistic cluster analysis.

The second step is to realize laboratory analysis which allows measurement of genetic distances between chosen chilli pepper varieties. For that purpose was chosen recent IPBS (inter-Primer Binding Site) method and statistic cluster analysis as well. Final result serves to confirm/refute following hypotheses:

Hypothesis:

$H^1$: All varieties of analysed 11 chilli peppers belong to 11 different species.

$H^2$: There is high similarity between results of morphologic and genetic results for each of analysed chilli peppers varieties.

$H^3$: Charapita amarillo chilli pepper can be substituted by other analysed variety according to its characteristics.

# 5 Materials and methods

## 5.1. Study area

Present research was realized in area near Pucallpa, Ucayali Department, Peruvian Amazon (Figure 3). There is a hot and humid topical climate (climate of lowland tropical rainforest). The average temperature is 25,7°C (with that rate range from 19,5 °C to 31°C). The relative air humidity rises up to 80% and the rainfall fluctuates in the scale from 1500 to 2100 mm/year. The climate is characterized by two rainy seasons (February-May) and the second one from September to November. The local altitude is 145, but also up to 300 masl. (Vicha, 2008; MINAG, 2002)


Figure 3. Peruvian region of Ucayali and capital city of the region - Pucallpa.

## 5.2. Study site

Material and data collection was realized in Peruvian Ucayali region in area of Pucallpa city in villages of Campo Verde district (Figure 4.). Campo Verde is located 31 km from Pucallpa city on Federico Basadre`s main road. There is a Campo Verde municipality, basic hospital, primary school, local market for small scale farmers, petrol station and local sport club. Campo Verde is characterized by undulated terrain in latitude of 145 meters above sea level, average temperature 25°C and approximately 40% of air humidity. Main agricultural activities are extensive cattle and poultry breeding and crop production (rice, corn, cassava,

citruses, plantains and other fruits). The soil is widely eroded and degraded by cattle and shifting cultivation. In villages of Pimental (8 km from Campo Verde) and Agua Blanca (6 km far from Campo Verde) there are established agroforestry systems, managed by APE Pimental association, which applies ecological agriculture. Material for this thesis was collected on APE Pimental plantations.

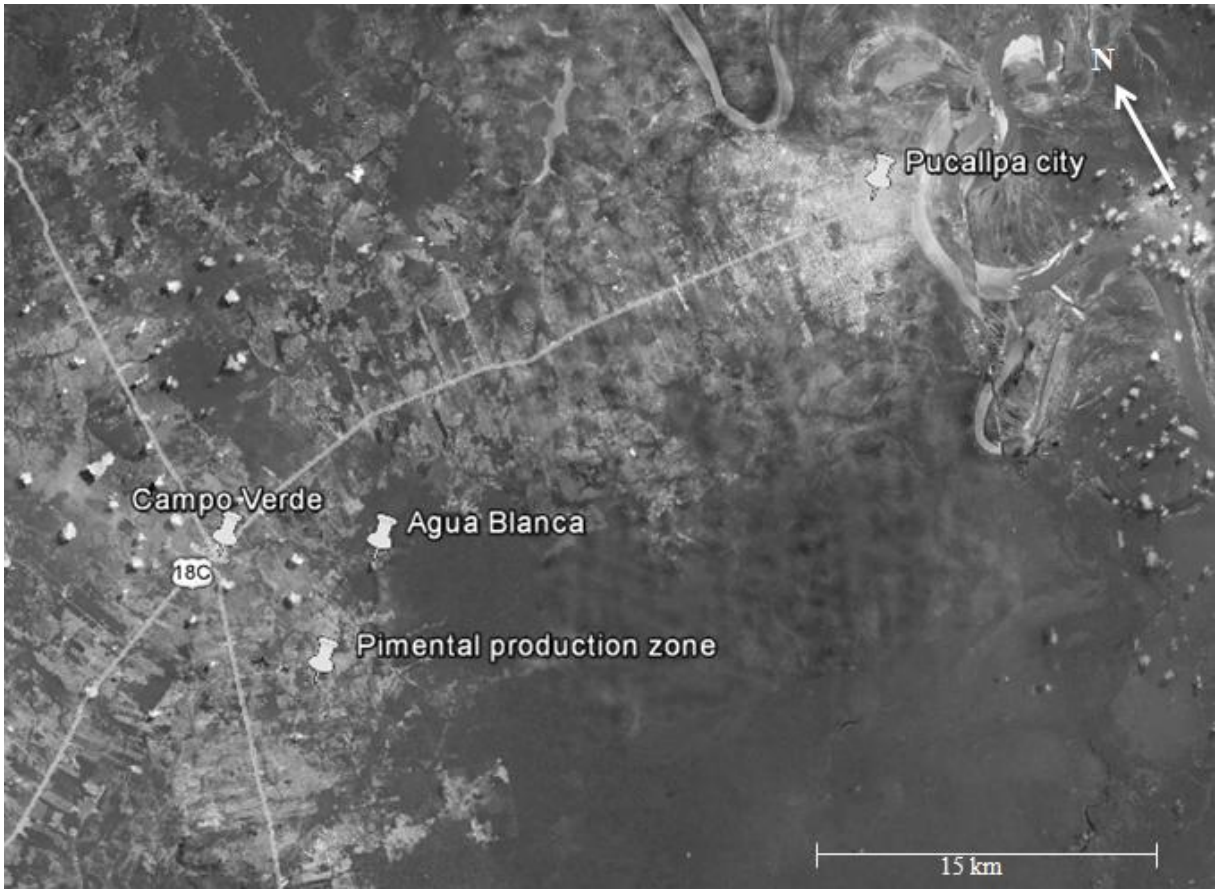


Figure 4. Campo Verde and Pimental production zone.

## 5.3. Collection of data, material and manipulation

### 5.3.1. Biological material and fruit harvest

For data collection were chosen 11 native chilli peppers (Table 1.) with high local and national economic and cultural importance.

Table 1. List of chilli peppers chosen for the investigation.

| Sample number | Local name | Scientific name[1] | Harvest date |
|---|---|---|---|
| 1 | Charapita rojo | *Capsicum chinense* Jacq | 13.9.2012 |
| 2 | Ayuyo | *Capsicum baccatum* | 13.9.2012 |
| 3 | Aji Charapita amarillo | *Capsicum chinense* Jacq | 13.9.2012 |
| 4 | Trompito rojo | *Capsicum* sp | 13.9.2012 |
| 5 | Trompito amarillo | *Capsicum* sp | 14.9.2012 |
| 6 | Cerecito rojo | *Capsicum* sp | 13.9.2012 |
| 7 | Cerecito amarillo | *Capsicum* sp | 13.9.2012 |
| 8 | Malagueta | *Capsicum frutescens* | 20.9.2012 |
| 9 | Challuaruro | *Capsicum baccatum* | 13.9.2012 |
| 10 | Upia ucho | *Capsicum* sp | 13.9.2012 |
| 11 | Pinchito de Mono (Pipi) | *Capsicum frutescens* | 13.9.2012 |

** Scientific names published in Ugas et al.(2000). Numbers of collected chillies correspond to numbers in figure 2.*

Collection of the material, fruits of 11 native chilli peppers (*Capsicum* sp.) was realized in September of 2012. The best and optimally mature fruits were found on ecological fields of APE Pimental Association, therefore were selected five different chilli peppers plantations (respectively plantations of five different producers with a distance minimally 1 km) to each other. There were randomly collected 50 fruits of each chilli type per plantation – (totally of five plantations 250 fruits of each type of chilli). The collected fruits were transported to Pucallpa city in boxes labeled by type, date and locality.

### 5.3.2. Morphological data collection

In each variety were collected following morphological data about the fruit:

- Fruit color at intermediate stage
- Fruit color at mature stage
- Fruit shape
- Fruit length [cm]
- Fruit width [cm]
- Fruit weight [g]
- Neck at base of fruit
- Fruit surface
- Seed color
- Number of seeds per fruit

According to IPGRI (1995) these mentioned characteristics are classified as highly discriminative descriptors for *Capsicum* species and are detail described in following sub-chapter as descriptors.

Data of capsaicin content were collected from published researches which were using fruits from Campo Verde district (Meckelmann *et al*., 2013). The length was taken using millimeter paper and the weighing was realized using sensible digital balance (accuracy: 0,001). Fruits`s shape and color was described according to published standardized descriptors (IPGRI, 1995). Seed number per fruit was recorded at the moment of manipulation with the fruits to provide biological material for laboratory analysis. All of these data were introduced to MS Excel 2003 for further statistical analysis.

### 5.3.3. Morphological descriptors used for this research

For morphological description was used publication titled "Descriptors for *Capsicum* (*Capsicum* spp.) issued by International Plant Genetic Resources Institute in Rome (IPGRI, 1995), which serves as internationally standardized descriptor. IPGRI (1995) recommends as specific reference for colors serves the RHS CCH scale for "yellow-to-red" colors in Appendix II.

**Fruit color at intermediate stage**

Recorded on fruits just before the ripening stage

1 White
2 Yellow
3 Green
4 Orange
5 Purple
6 Deep purple
7 Other (specify)

**Fruit color at mature stage**

1 White
2 Lemon-yellow
3 Pale orange-yellow
4 Orange-yellow
5 Pale orange
6 Orange
7 Light red
8 Red
9 Dark red
10 Purple
11 Brown
12 Black
13 Other (specify)

**Fruit shape**

1 Elongate
2 Almost round
3 Triangular
4 Campanulate
5 Blocky
6 Other (specify)

As specific reference serves figure 5.

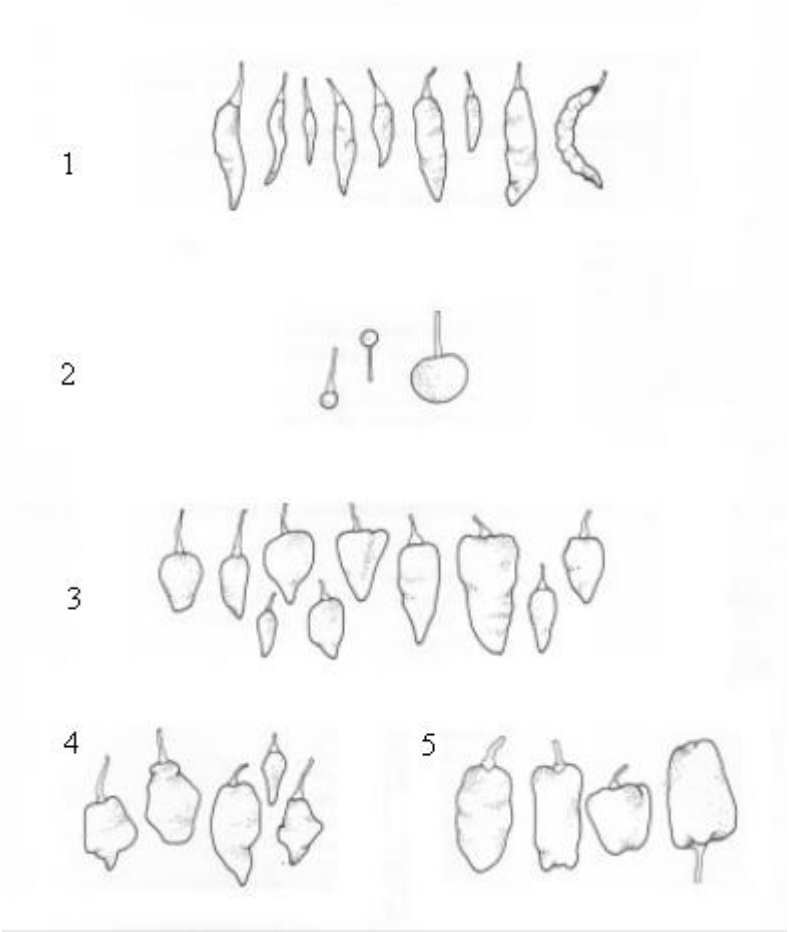


Figure 5. Types of fruit shape.

**Fruit length** [cm]

Average fruit length of 10 ripe fruits of the second harvest

**Fruit width** [cm]

Measured at the widest point. Average fruit width of 10 ripe fruits of the second harvest

**Fruit weight** [g]

Average fruit weight of 10 ripe fruits of the second harvest

**Neck at base of fruit**

0 Absent

1 Present

As specific reference serves figure 6.


Figure 6. Neck at base of the fruit (0 – absent, 1 – present).

**Fruit surface**

1 Smooth

2 Semi-wrinkled

3 Wrinkled

**Seed color**

1 Straw (deep yellow)

2 Brown

3 Black

4 Other (specify)

**Number of seeds per fruit**

Average of at least 10 fruits selected from 10 random plants

1 <20

2 20-50

3 >50

### 5.3.4. Descriptor data evaluation

All recorded data were entered to MS Excel 2003 table for further analysis on PAST version 2.17c statistic programme. As the result the dendrogram is required.

## 5.4. Analysis in laboratory conditions

The entire fruits were washed in water and dried separately on direct sunshine for three days. After drying, there was realized the seed separation from the fruit pulp in sterile conditions, using sterile glows and sterilized knife. Only good seeds (without signs of infection or putridity) were selected and additionally dried again on direct sunshine to prevent rottenness. Dry seeds of each chilli were divided in three parts and packed in three clean paper envelopes; then were numbered (Table 1.) and labeled by chilli pepper`s name, date and locality of origin. The same sample numbers are used during whole research.

### 5.4.1. Preparation of DNA material

First was needed to purify the DNA. Material of DNA isolation was prepared directly from the seeds of analyzed chilli peppers. Seeds were homogenized using liquid nitrogen and then the DNA as isolated by "GeneJET™" Plant Genomic DNA Purification Mini Kit (Thermo Scientific) in figure 7 according to recommended protocol (Apendix I.).


Figure 7. GeneJET™ DNA purification kit

Quantity of isolated DNA was determined using Quibit™ Fluorometer Invitrogen device according to producers recommendation (Figure 8.). The Qubit® 2.0 Fluorometer utilizes specifically designed fluorometric technology using Molecular Probes® dyes to quantitate biomolecules of interest. These fluorescent dyes emit signals only when bound to specific target molecules, even at low concentrations.


Figure 8. Quibit™ Fluorometer Invitrogen

### 5.4.2. Condition optimization of polymerase chain reaction for iPBS analysis

In reaction mixture, there were optimized concentration of single components to determine suitable conditions of polymerase chain reaction for iPBS analysis.

1) To determine optimal DNA amount were used following amounts of isolated DNA of Ayuyo genotype: 10; 20; 50 a 100 ng / 15 µl of reaction mixture.
2) To determine optimal primers concentration were used concentrations of the primer N° 1847: 200, 400, 600 nmol.dm$^{-3}$.
3) The temperature gradient from 49 to 59 °C was tested to determine the optimal temperature for primer binding.

All optimization reactions were repeated three times to prove result repeatability of iPBS analysis. In polymerase chain reaction, there were also tested the negative control-samples without DNA to determine the effect of components concentration on primer multimers synthesis in reaction mixture.

### 5.4.3. Primers and iPBS analysis

The collection of 15 iPBS primers was analyzed according to Kalender et al. (2010). Three of them provide monomorphic profiles in all tested genotypes and eight don't provide enough reproducible iPBS profile in realized triplicates. For analysis were used 5 iPBS markers (Table 2).

Table 2. Nucleotide sequence of primers (iPBS)

| Primer code[1] | Nucleotide sequence |
|---|---|
| 1899 | tgagttgcaggtccaggcatca |
| 1867 | tcgacttgaatccgctgctgcca |
| 1847 | ccagtccgaactacaatggccgggt |
| 1833 | cttgctggaaagtgtgtgagagg |
| 2078 | gcggagtcgcca |

[1]Primer codes are described according to Kalender *et al*. (2010).

Conditions for iPBS analysis were determined on the basis of the optimization of polymerase chain reaction as mentioned here above.. All reactions were realized in 15 µl reaction mixture containing: 1x Dream Taq PCR MasterMix (Thermo Scientific), 400 nmol.dm$^{-3}$ primer and 50 ng entire genomic DNA in MyCycler™ (Biorad) device. Time and temperature profile was as follows: 3 minutes at 95 °C, (30 seconds at 95°C, 60 seconds at 52°C, 2 minutes at 72 °C) 44-times and 7 minutes at 72 °C.

### 5.4.4. Genetic data evaluation methods

Digital image of electrophoreogram was evaluated using GeneBox System. Then the MS Excel 2003 was used to analyze background data for presence of absence of the fragment in electrophoreogram track. That formed the basis for hierarchical clustering (cluster analysis which seeks to build a hierarchy of clusters by UPGMA method. Using this method in SYNTAX programme was determined average Euclidean distance of clusters and the branch-structure of clusters was done (dendrogram). The background data of the fragment presence or absence were used also to calculate the Jaccard index "Jaccard similarity coefficient" (Jaccard 1991).

# 6 Results and discussion

## 6.1. Morphological analysis

First, there were collected data of morphological characteristics of chosen chilli peppers according to values and scales of internationally standardized morphological descriptors (IPGRI, 1995). Data were inserted to MS Excel 2003 and sorted in qualitative and quantitative characteristics according to fruit and seed. (Tables 3 and 4). *(RHS color scale description – Apendix II. - serves as additional information and wasn't processed in statistics.)*

Table 3. Morphological characteristics of chilli pepper fruit.

| | Fruit | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Qualitative | | | | | | Quantitative | | | |
| Local Name | Shape | Neck at base of fruit | Color / mature stage | Clor RHS/ mature stage | Color / intermediate stage | Surface | Diameter (cm) | Length (cm) | Weigth (gr) | Capsaicin content (mg/100gr) |
| Charapita rojo | 2 | 0 | 9 | 34A | 3 | 1 | 1,1 | 0,8 | 0,41 | 190 |
| Ayuyo | 5 | 0 | 7 | 33A | 1 | 1 | 1,7 | 5,8 | 2,40 | 170 |
| Charapita amarillo | 2 | 0 | 4 | 9A | 3 | 1 | 1,1 | 0,8 | 0,29 | 424 |
| Trompito rojo | 3 | 0 | 7 | 33A | 2 | 1 | 1,7 | 2,0 | 1,95 | 320 |
| Trompito amarillo | 3 | 0 | 2 | 3A | 3 | 1 | 1,1 | 1,7 | 0,80 | 429 |
| Cerecito rojo | 2 | 0 | 9 | 46C | 3 | 2 | 1,5 | 1,4 | 3,90 | 400 |
| Cerecito amarillo | 2 | 0 | 3 | 14B | 3 | 2 | 1,8 | 1,1 | 3,50 | 300 |
| Malagueta | 1 | 1 | 6 | 28B | 3 | 1 | 1,5 | 5,0 | 1,45 | 1530 |
| Challuaruro | 1 | 1 | 7 | N30A | 1 | 1 | 1,4 | 4,8 | 2,40 | 280 |
| Upia ucho | 5 | 1 | 8 | 40B | 3 | 2 | 1,0 | 3,0 | 1,40 | 1170 |
| Pinchito de mono | 1 | 1 | 7 | 40B | 3 | 1 | 0,5 | 1,9 | 0,40 | 995 |

Table 4. Morphological characteristics of chilli pepper seed.

| | | Seed | | |
|---|---|---|---|---|
| | | Qualitative | | Quantitative |
| Species number | Local Name | Colour | Colour RHS | N° fo seeds per fruit |
| **1** | Charapita rojo | 1 | 2D | 1 |
| **2** | Ayuyo | 1 | 3D | 2 |
| **3** | Charapita amarillo | 1 | 2D | 1 |
| **4** | Trompito rojo | 1 | 3D | 1 |
| **5** | Trompito amarillo | 1 | 2D | 1 |
| **6** | Cerecito rojo | 1 | 3D | 2 |
| **7** | Cerecito amarillo | 1 | 2D | 2 |
| **8** | Malagueta | 1 | 3D | 2 |
| **9** | Challuaruro | 1 | 3D | 2 |
| **10** | Upia ucho | 1 | 3D | 1 |
| **11** | Pinchito de mono | 1 | 3D | 1 |

Among other data, the charapita amarillo (3) presents similar qualitative data and capsaicin contents as cerecito rojo (6) and cerecito amarillo (5). The malagueta (8) and upia ucho (10)

shows extreme content of capsaicin (table 4). The challuaruro (9) and ayuyo (2) has similar content of capsaicin and the same color at mature stage. The statistical analysis concludes morphological data.

The following dendrogram was generated by statistical programme PAST version 2.17c using classic cluster analysis with standardized Euclidian distance (Figure 9.).

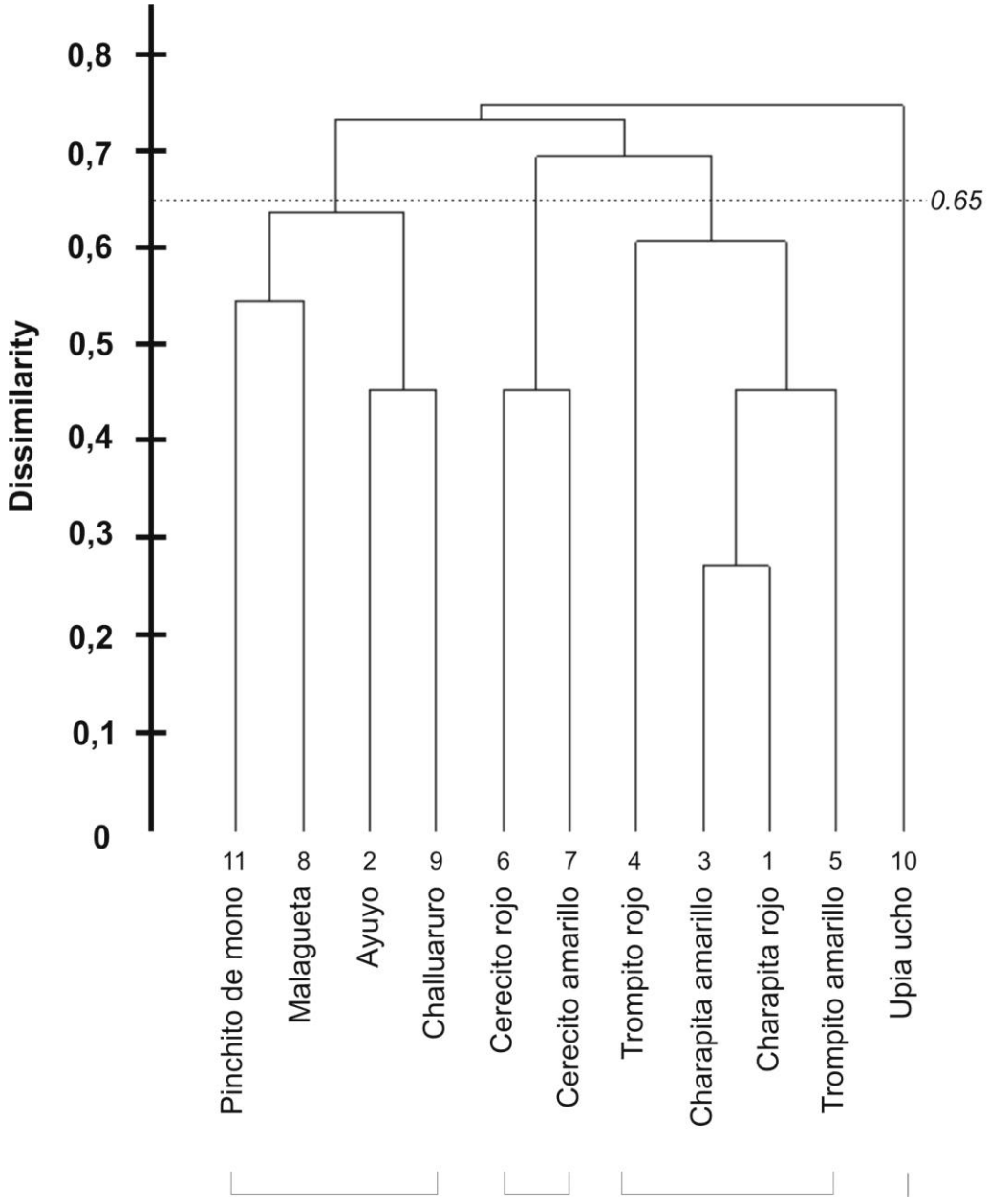


Figure 9. Cluster analysis of morphological characteristics of 11 chosen chilli peppers.

As the dendrogram dissimilarity scale shows, there are four main groups at the level of 65 % of dissimilarity. The first group includes varieties of pinchito de mono (11), malagueta (8), ayuyo (2) and challuaruro (9). All of these have elongated shape and are red at mature stage. The first two chillies (11 and 8) have high average content of capsaicin ( 1000 – 1200 mg / 100 g), so it is up to ten times higher than in other two chillies (2 and 9) with average capsaicin content of 225 mg / 100 g. These two are also purple colored at premature stage.

Then there are shown two cerecito chillies with very similar weight and capsaicin content. The third group is formed by charapita amarillo (3), charapita rojo (1) then trompito amarillo (5) and trompito rojo (4). The charapita chllies has very low dissimilarity rate (0,28) and are characterized by small round fruits. The trompitos are characterized by triangular fruit shape. Both varieties have red and yellow color type. Last chilli is upia ucho (10) with small red fruit, high capsaicin content is statistically sorted alone as fourth group at highest dissimilarity rate of 0,76.

Analysis of morphologic charatersitics can be affected by wide scale of values in data of capsaicin content but the varieties are sorted according to their shape, size and colour what correspond. Groups and variety relation presented in the graph here above correspond to the ay ho the local producers sort chilli plants which is underlined by similarities in local native names of the plants.

For exact result is needed the comparison to genetical analysis.

## 6.2. Genetical analysis

The results of genetical analysis were expressed in dendrogram type of graph (Figure 10).

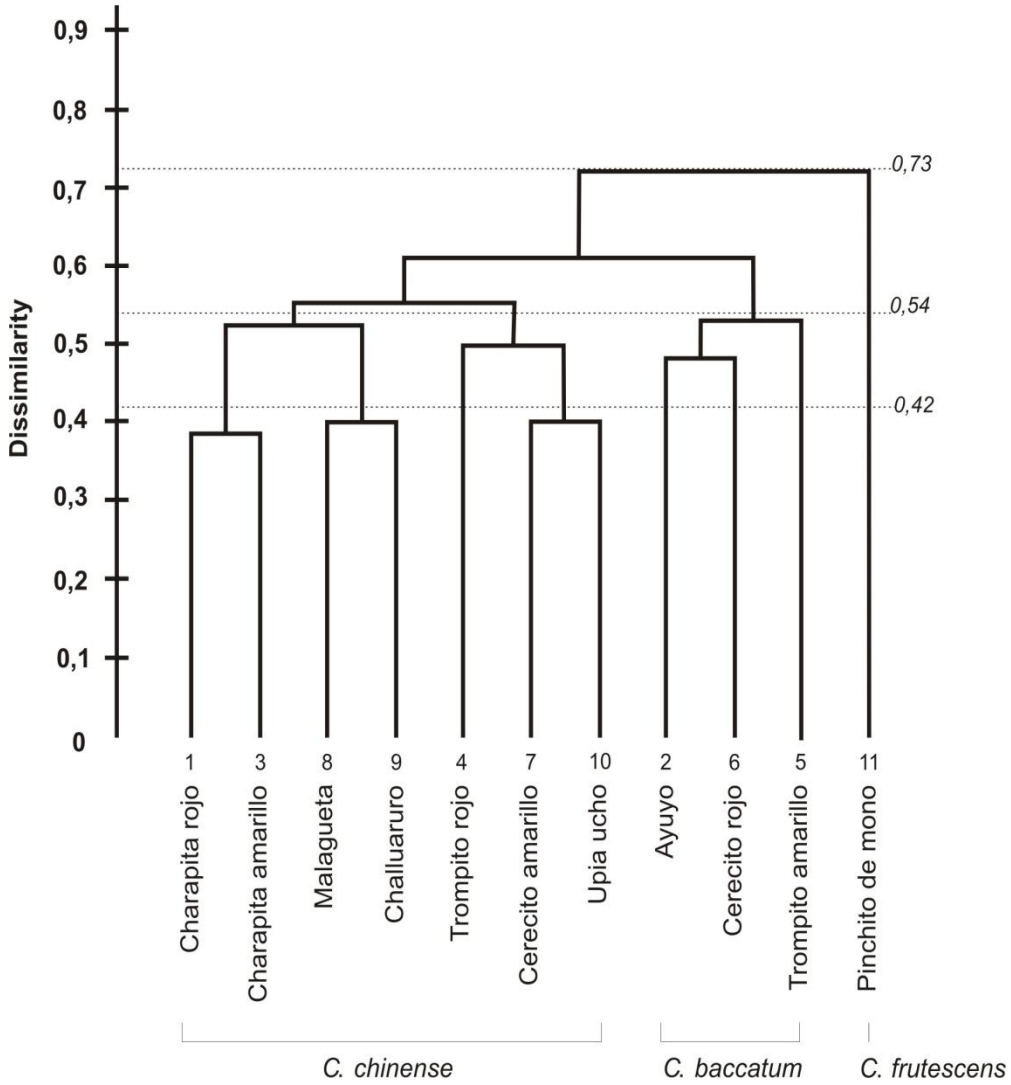


Figure 10. Cluster analysis of genetic characteristics of 11 chosen chilli peppers.

As the results show, at the level of 54% of dissimilarity, there can be considered four groups (clusters) of chili peppers. The first group includes the charapita amarillo (3) chilli which is preferred by local people in Peruvian Amazonia. This chilli presents a 38% of dissimilarity to charapita rojo (1) forming a small sub-group of small rounded chilli peppers. Ugaz *et al*. (2000) who had done a research of chilli peppers based on morphological descriptors, state that charapita chillies (1 and 3) belong as varieties to *Capsicum chinense* species. Our results can confirm its close genetic relation. In the graph here above is clear that other two chilli peppers malagueta (8) and challuaruro (9) are genetically related at 40% of dissimilarity to

each other and at 0,53 of genetic dissimilarity level are grouped to the charapita chillies (1,3). It is supposed to be varieties of the same species (*C. chinense*) because it doesn't present genetical difference more than 60%. But this result doesn't correspond to Ugaz *et al*. (2000) who classified malagueta chilli (8) as *Capsicum frutescens* and the challuaruro (9) was determined as *Capsicum baccatum* by the same author.

The second group shown on the figure 10 is composed of trompitos rojo (4), cerecito amarillo (7) and upia ucho (10), which are bind together by graphic line at level of 50% of dissimilarity and are genetically connected to the first group at 56% dissimilarity level. These three varietis of chilli peppers doesn't pose in scientific literature as determined varieties of already described species. In the present study they are included as varieties of *Capsicum chinense* species for its genetic relation to charapita chillies (1 and 3).

The third group is formed by ayuyo (2), cerecito rojo (6) and trompito amarillo (5) and is bind to *C. chinense* group at dissimilarity higher than 60% exactly at 63% which supposedly doesn't form varieties of the same species. This group present Ayuyo chilli pepper (2) scientifically determined as variety of *C. baccatum* species (Ugaz *et al*. 2000). The chillies 6 and 5 are bind to Ayuyo (2) at 54% of dissimilarity and in present work are included under *C. baccatum* species as its varieties.

At least, there is a fourth group formed by only one chilli pepper: pinchito de mono (11). This chilli is bind to other groups at dissimilarity level of 0,73 which makes it genetically very different to other analyzed chilies. This chilli is considered to be variety of *C. frutescens* species, known in Amazonia for its tiny size and high ardor (Ugaz *et al*., 2000 and APEGA, 2009).

Comparing the morphologic and genetic analysis can be observed interesting difference in grouping of cerecito chillies (6 and 7) and trompito chilies (4 and 5), according to morphological analysis there are closely related, but it doesn't correspond to genetic analysis which sort them in another group.

# 8 Conclusions

We conclude that Pimental production zone in Campo Verde district of Ucayali is quite diverse in native chilli peppers. On the basis of descriptive data of eleven chilli peppers collected in this area was done morphologic description and statistical analysis which results in grouping these varieties under specific clusters.

The following genetical research using modern IPBS method allowed us the measurement of genetic distance between analyzed chilli varieties. According to measured genetical distances in statistical programs we can conclude the presence of three species among studied varieties: *Capsicum chinense, C. baccatum* and *C. frutescens* what refuses our first hypothesis.

Comparing our second hypothesis to results of morphological and genetical analysis we conclude following fact: there is a high similarity between morphological and genetical characteristics in charapita amarillo and charapita rojo, which presents was directly bind in both cluster analysis and in both of them presents the lowest dissimilarity rates. In case of other 9 analyzed chilli peppers there is no morphological binding pair identical to genetical clusters.

The chilli charapita amarillo has high local economic and cultural importance. According to morphological characterization, the best substitute for charapita amarillo can be the cerecito rojo and trompito amarillo with very similar content of capsaicin and (average mg 400 mg/100g) and bigger fruit size. Comparing theoretical results to data from practice and production we can conclude trompitos amarillo the best substitute because of its high yield (up to 20 t / ha). The cerecito rojo produces only 5,5 t/ha, so the half of normal yield of charapita amarillo.

This thesis represents a good base of date for further investigation and studies in field of genetics and problematic of plant species with high economic and cultural importance.

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# Appendices

## Appendix I.

*This chapter was prepared and cited according to official protocol issued on websites of Thermo Scientific Company accessible at:* http://www.thermoscientificbio.com/nucleic-acid-purification/genejet-plant-genomic-dna-purification-mini-kit/?rdr=true&terms=plant+dna+purification+mini+kit

### A. Plant Genomic DNA Purification Main Protocol

STEP 1. Pipette 350 µl of Lysis Buffer A into 1.5 ml microcentrifuge tube. Weigh the plant tissue - use up to 100 mg of fresh or frozen tissue; up to 20 mg of lyophilized tissue. Grind the material by one of the following methods:

a) Mortar and pestle.

Place up to 100 mg of plant tissue into liquid nitrogen and grind thoroughly with a mortar and pestle.

b) Grinding mill. Place up to 100 mg of tissue into a vial containing stainless steel beads. The vial and beads should be precooled with liquid nitrogen. The setup of the mechanical disruption depends on the tissue type. Immediately transfer the tissue powder into a 1.5 ml microcentrifuge tube containing 350 µl of Lysis Buffer A. Vortex for 10-20 s to mix thoroughly.

*Note:*

*• Transfer the ground tissue to the Lysis Buffer as quickly as possible to avoid DNA degradation.*

*• All ground material must be thoroughly mixed with the Lysis Buffer. DNA degradation can occur in particles that are left to dry on the walls of the tube.*

*• Ground tissue can be used immediately in the DNA isolation protocol or stored at -70°C until use.*

STEP 2. Add 50 µl of Lysis Buffer B and 20 µl RNase A. Optional: for tissues that are resistant to mechanical disruption, add glass sand to the microcentrifuge tube and vortex for 1 min.

STEP 3. Incubate the sample 10 min at 65°C vortexing occasionally or use a shaking water bath, rocking platform or thermomixer.

STEP 4. Add 130 µl of Precipitation Solution and mix by inverting the tube 2-3 times. Incubate 5 min on ice.

STEP 5. Centrifuge for 5 min at ≥20,000 x g (≥14,000 rpm).

STEP 6. Collect the supernatant (usually 450-550 µl) and transfer to the clean microcentrifuge tube (not provided). Add 400 µl of Plant gDNA Binding Solution and 400 µl of 96% ethanol and mix well.

**B. Protocol model of DNA purification of seeds in steps**

STEP 1. In a 1.5 ml microcentrifuge tube add 350 µl of Lysis Buffer A supplemented with dithiothreitol (DTT) to a 40 mM final concentration. Grind up to 100 mg of plant material in liquid nitrogen using a mortar and pestle or grinding mill as described in Step 1 on p.4.

STEP 2. Transfer the ground plant tissue powder into tubes containing the prealiquoted Lysis Buffer A supplemented with DTT. Add 50 µl of Lysis Buffer B and 20 µl RNase A. Mix by vortexing or pipetting. Optional: for tissues resistant to mechanical disruption, add glass sand to the microcentrifuge tube and vortex for 1 minute.

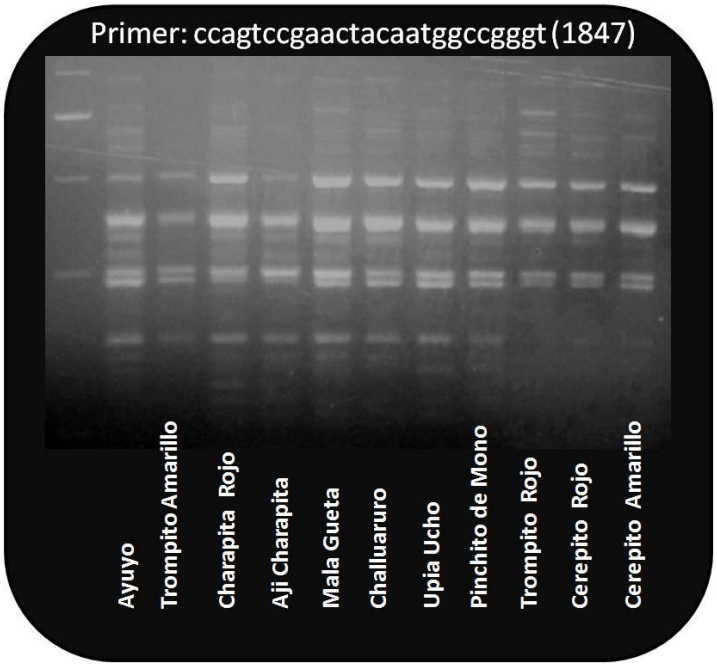
STEP 3. Proceed to step 3 on p.4 of the Plant Genomic DNA Purification Main Protocol.

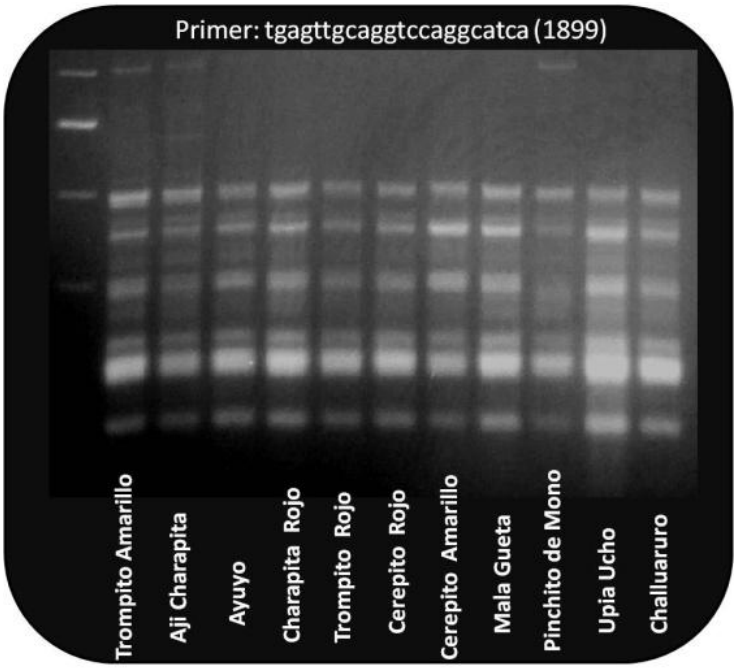
## Appendix II. Standardized color scale.

http://rhscf.orgfree.com/a.html

## Appendix III. Polymorphic bands.

Samples of polymorphic bands of IPBS analysis



**Appendix IV. Additional photos of analyzed native chilli peppers.**

Carapita rojo (1)

Ayuyo (2) – at premature stage.

Charapita amarillo (3) fruits and plantation

Trompito rojo (4)

Trompito amarillo (5)

Cerecito rojo (6)

Cerecito amarillo (7)

Malagueta (8)

Challuaruro (9) – red mature fruit, yellow/purple pre-mature fruit

Upia ucho (10)

Pinchito de mono (11)