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Faculty of Tropical AgriSciences

Department of Crop Sciences and Agroforestry

Phenotypical and genetic variability of *Myrciaria dubia* in Peruvian Amazon

M.Sc. thesis

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CZECH UNIVERSITY OF LIFE SCIENCES PRAGUE

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DIPLOMA THESIS ASSIGNMENT

Bc. Klára Čermáková

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Thesis title

Phenotypical and genetic variability of Myrciaria dubia in Peruvian Amazon

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Objectives of thesis

The camu-camu (Myrciaria dubia) is currently one of the most important fruit spices that grows in area of the Peruvian Amazon, because of high content of vitamin C. That puts camu-camu high on the market. The main objective is to determine genetic and nutritional variability of camu-camu in Peruvian Amazon. The specific objectives are: to assess intra- and interpopulation genetic diversity of wild and cultivated populations of camu-camu, to find out if there is connection between content of vitamin C in fruits and genetics. The theses will be focus on variability(differences) of cultivated and natural populations. This research could also help us determine the approximate origin of cultivated populations. The results of the research could lead to further improvement of domestication and breeding of that species.

Methodology

There will be sampling around area of Iquitos (natural populations) and Pucallpa (cultivated populations) in Peru. For genetic analysis, we plan to select 10-20 natural populations from the experimental field of IIAP near Iquitos. The populations will be chosen from different watersheds in the Peruvian Amazon and some populations will be from Brasil. Cultivated populations will be sampled around Pucallpa city on several plantations. The content of the vitamin C in the fruit will be determined by illman's method in the laboratory of Universidad Nacional de la Amazonia Peruana. Genetic diversity among and between these populations will be analysed using microsatellite markers (SSR markers) when we use 6 fluorescently labeled primers. The length of the microsatellites will be measured by capillary electrophoresis. Genetic analysis will be performed in the laboratory of CULS and data will be processed in software Fstat and Structure. All data will be statistically analyzed.

This research will help us determine the approximate origin of cultivated populations and improvement in further breeding.

The proposed extent of the thesis

40-60 pages

Keywords

camu-camu, genetic diversity, population genetics, vitamin C, PCR

Recommended information sources

Penn, James W. 2008."Non–timber Forest Products in Peruvian Amazonia: Changing Patterns of Economic Exploitation. FOCUS on Geography 51.2 : 18-25.

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DECLARATION

I, Klára Čermáková, hereby declare that this thesis, submitted in partial fulfilment of requirements for the master's degree in Faculty of Tropical AgriSciences of the Czech University of Life Sciences Prague, is wholly my own work written exclusively with the use of the quoted sources

In Prague, 27 April 2018

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Bc. Klára Čermáková

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ABSTRACT

Camu-camu (*Myrciaria dubia*, McVaugh) is one of the most important fruit species from South America. The production is focused on the Peruvian Amazon, Brazil or Bolivia, where this tree naturally occurs. In recent years, the production of this fruit has increased due to an increase in demand on the international market. This species is particularly important for the high content of vitamin C and the high proportion of antioxidants. To cover such a great demand, the first plantations were established in 1998 and the breeding process, which is still at the beginning, began. Most farmers use undomesticated plants, usually taken from the wild nature or just made basic selections, when selecting the best profitable individuals. The aim of this work was to characterize the genetic variability of both wild and cultivated populations in the Peruvian Amazon. The analysis focused on diversity within and between the population. Another part of this work was to find out if there is any correlation between the amount of vitamin C in the fruits and the origin or properties transferred in the genetic information. This work is based on the sampling of 358 individuals of 31 populations, 21 of these populations being wild and originating around the city of Iquitos; two populations were collected as representatives of cultivated populations also around that city, and the remaining eight were collected from the plantations around Pucallpa. For the genetic analysis, 10-15 individuals were randomly selected from each of the population and samples of leaves were taken from each one (n = 357). For analysis of vitamin C content, fruits were collected from 53 different individuals belonging to 25 different populations. For the detection of genetic diversity, six SSR primers have been used, which have already been developed and used for a previous study at our university. By using computer programs, the main variability indexes and the related dendrograms were created. The modified Tillman Volumetric method was used to determine the ascorbic acid content in the fruits. This analysis was carried out in the laboratory of the Peruvian University of the Universidad Nacional de la Amazonia Peruana. For determination if there is influence of genetic diversity to content of vitamin C in the fruit was used AMOVA, to calculate the percentage of variance among and within population of the total molecular variance. The variance within populations was 99% and among populations only 1%.

Keywords: camu-camu, PCR, population genetics, SSR primers, vitamin C

ABSTRAKT

Camu-camu (Myrciaria dubia, McVaugh) je jedním z nejdůležitějších ovocných druhů pocházejících z Jižní Ameriky. Jeho produkce je soustřeďována na peruánskou Amazonii, Brazílii či Bolívii, kde se přirozeně vyskytuje. V posledních letech se zvýšila produkce tohoto ovoce díky navýšení poptávky na mezinárodním trhu. Tento druh je hlavně významný z důvodu vysokého obsahu vitamínu C a velkého podílu antioxidačních látek. Aby se pokryla tak velká poptávka byly založeny první plantáže v roce 1998 a s tím začal i proces šlechtění, který je zatím stále na začátku. Většina farmářů využívá nešlechtěné rostliny získané sběrem z přírody nebo prošli jen základní selekcí, kdy jsou vybrány nejlépe profitující jedince. Cílem této práce bylo charakterizovat genetickou variabilitu jak divokých, tak pěstovaných populací v peruánské Amazonii. Analýza byla zaměřena na diverzitu v rámci populace i mezi nimi. Jako další část této práce bylo zjistit, zda je souvislost mezi množstvím vitamínu C v plodech a původem či vlastnosti předávanými v genetické informaci. Tato práce vychází ze sběru 31 populací, kdy 21 z těchto populací bylo divokých a pocházely z oblasti kolem města Iquitos. Dvě populace byly sebrány jako zástupci pěstovaných populací také okolo toho města, zbylých osm bylo posbíráno z plantáží kolem města Pucallpy. Pro genetickou analýzu bylo náhodně vybráno 10-15 jedinců z každé populace, z nichž byly odebrány vzorky listů (n=357). Pro analýzu obsahu vitamínu C byly sesbírány plody z 53 různých jedinců patřících do 27 odlišných populací. Ke zjištění genetické diverzity bylo použito 6 SSR primerů, které již byly vytvořeny a použity na předchozí studii na naší univerzitě. Použitím počítačových programů pak byly určeny hlavní indexy variability a vytvořeny dendrogramy příbuznosti. Na zjištění obsahu kyseliny askorbové v plodech byla použita modifikovaná Tillmanova metoda. Tato analýza proběhla v laboratoři peruánské univerzity Universidad Nacional de la Amazonia Peruana. K určení, zda existuje vliv genetické diverzity na obsah vitaminu C v ovoci, byla použita AMOVA pro výpočet molekulárí variace mezi populací a uvnitř populace. Rozdíly v populacích byly 99 % a u populací pouze 1 %.

Klíčová slova: camu-camu, PCR, populační genetika, SSR primery, vitamín C

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2.1 Camu camu (*Myrciaria dubia)*

Camu-Camu (*Myrciaria dubia,* (H.B.K.) Mc Vaugh) is a small shrub that reaches around 3 m in height, it belongs to the family Myrtaceae. The highest concentration of natural populations is found around the Amazonas and Ucayali rivers, especially between cities of Pucallpa and Iquitos in Peru. It can be naturally found also on floodplains of Brazil, Columbia or Venezuela (Villachica 1996; Fernández et al. 2001). The genus *Myrciaria* includes around 120 species, which are used as food or in traditional medicine (IPNI 2005).

2.1.1 Botanical description

M. dubia is a shrub that is usually around 3 m high but reaches up to 8meters. It is a deeply rooted plant and has smooth light brown bark with grey spots. The branches grow from the base and individual branches can develop up to 15cm in diameter (Figure 1). On the trunk, during the dry season, the lamellae can be observed. They appear when the water drops down and the skin peels off (Villachica 1996).

Leaves are ovate-elliptic to lanceolate from 4.5 to 12.0 cm long and 1.5 to 4.5 cm wide, the leaf apex is acuminate, margin entire and slightly wavy (Figure 2). Axillary inflorescence with four subsessile hermaphrodite flowers are arranged in two pairs with rounded and ciliated bracts and small white perfumed petals (Figure 3). These flowers have about 125 stamens which are 6 to 10 mm long.

The fruit is a globular berry, from 10 to 32 mm in diameter. The colour is from green to dark violet, depending on the state on ripeness (Figure 4). The pulp inside is soft, juicy, extremely acid and pink with one to three 8 to 15 mm long seeds, which are conspicuously flattened and covered by a mesh of fibrils (Figure 5) (Villachica 1996; Rodrigues et al. 2001).

temperature, nutrients influencing the biosynthesis of vitamin C and the level of flooding (Rodrigues et al. 2016a).

The limited knowledge about genetic diversity of M. dubia, thus the primary objective of this study was to evaluate the intra- and interpopulation genetic diversity of wild and cultivated populations population of M. dubia in Peruvian Amazon and to determine the influence of genotype on the vitamin C content in fruits of selected populations of camu-camu.

1. INTRODUCTION

The Amazon basin's tropical forest is the largest rain forest in the world counting about six million km². This area covers over six countries including Brazil, Peru, Bolivia, Columbia, Venezuela and Ecuador (Rodrigues & Marx 2006; Martin et al. 2014).

Such ecosystem counts large number of potentially lucrative plant species. Camu-camu (*Myrciaria dubia*, McVaugh) it one of the fruit bearing tree that grows naturally in the whole Amazonian basin (Rodrigues & Marx 2006). It is a shrub or small tree, 4-8 m tall from Myrtaceae family native to the floodplains of Amazonia. It is highly tolerant to flooding, withstanding 4 to 5 months with the roots and even one-half or two-thirds of the stem submerged in water (FAO 2018). This fruit has been used by locals for many years in juices, ice creams, liquors and other products. The fruits are valued for their high content of vitamin C. However, various studies give quite a high variation of the ascorbic acid content in range between 877 and 3,133 mg/100g of pulp, nevertheless the commercial interest in this species has been growing on national and also on international level (Pinedo et al. 2004; Martin 2014). Traditionally, the fruits have been collected from wild populations growing usually along rivers and oxbow lakes and the cultivation started only few decades ago, when the world demand rapidly increased (Martin et al. 2014).

The information about genetic diversity of this species is still low, as most of the research was focused on the ascorbic acid content (Zapata & Dufour 1993; Andrade et al. 1995; Justi et al. 2000; Chirinos et al. 2010), even though the knowledge of genetic diversity could be important for future domestication and breeding of new varieties which can achieve higher yield, higher vitamin C content or resistance to certain pests and diseases. These data are also important for gene mapping and for finding new subspecies.

Considering that this species grows in populations that are most likely isolated from each other and on such a big area, diversity among populations seems to be quite high (Koshikene 2009; Rojas et al. 2011, Šmíd et al. 2016). For assessing genetic diversity by DNA markers, SSR markers are the most convenient to use because they are present in the genome of all organisms and they are codominant (Rojas et al. 2011).

Content of vitamin C in the fruits is affected mostly by stage of maturity during the harvest (Yuyama 2002; Teixeira 2004; Iman et al. 2011; Pinedo 2012), but there may be other factor including variability on the ecotypes and genotypes, environmental factors, water and soil pH,

LIST OF ABBREVIATIONS

CT…………………………………...………………………… cytosine, thymine

CTAB…………………………...…………………cetyl trimethylammonium bromide

GA……………………………………………...…………...…guanine, adenine

PCR …………………………...…………………………polymerase chain reaction

RPM…………………………………...……………………………rotation per minute

SSR …………………………..……………………...…………simple sequence repeat

IIAP………………………… …..Instituto de Investigaciones de la Amazonía Peruana

AMOVA……………………………………………….. Analysis of molecular variance

INIA……………………………………...…Instituto Nacional de Innovación Agraria

RAPD…………………………….…………...…Random Amplified Polymorphic DNA

AFLP……………………….…………….…Amplified Fragment Lenght Polymorphism

ISSR……………………………………………….Inter Simple Sequence Repets

DCIP…………………………………………….....2,6-dichlorophenolindophenol

HPLC…………………………………….High Performance Liquid Chromatography
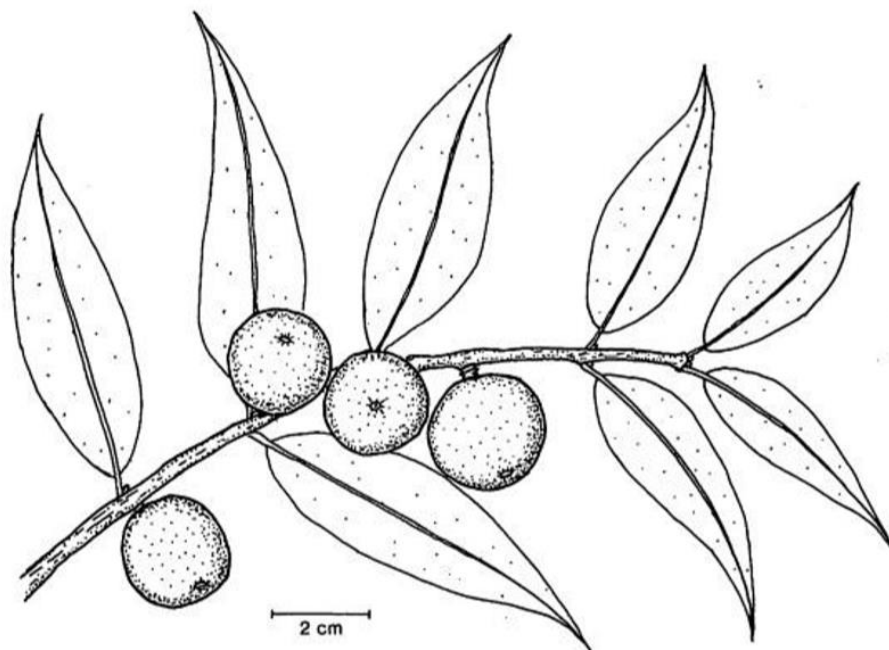
Figure 1. *Myrciaria dubia* McVaugh branch with fruits (Peters & Hammond 1990).

Figure 2. Leaves of camu-camu (author: Jan Šmíd).

Figure 3. Open flower and flower buds (author: Jan Šmíd).

Figure 4. Stages of ripeness. On the left is fully ripe fruit. Fruit with the highest content of Vitamin C is second from left. (author: Jan Šmíd).

Figure 5. Cross section of the fruit with visible seeds (author: Ing. Jan Šmíd).

2.1.2 Ecology

Camu-camu is native to the Amazon, especially the "Tropical Humid Forest" life zone, which is predominant in the Peruvian Amazon, where the specie develops in periodically flooded areas with high humidity. This species is flood-tolerant and can remain completely submersed in water for approximately 5 months (Rodrigues 2001).

The "Tropical Humid Forest" is characterized by range of temperatures from 22 °C to 32 °C, with the average of 26 °C. This range is ideal for camu-camu, since this plant develops rapidly in temperatures oscillating between 20 °C and 35 °C (Fernández et al. 2001; Rodrigues 2001).

The zones with wildly growing camu-camu have relatively high average rainfall, from 2500 to 3000mm/year, which is satisfactory to cover the water requirements of the species. The substrates also help with the source of water during the dry season, where camu-camu grows on alluvial soil with a muddy, clay-like, gritty texture and in only lightly drained soils that hold water. The values of pH in this type of soil vary between 4.5 and 6.5 (Fernández et al. 2001; Rodrigues 2001). The content of nutrients in the soil is usually extremely low (Clay & Clement 1993).

Since camu-camu is bound to water, the desirable altitude for cultivation tends to be low. In general, altitudes lower than 300 m a.s.l are considered adequate. There are no references to their behaviour at higher altitudes (Fernández et al. 2001).

2.1.3 Origin and distribution

The centre of origin of camu-camu is in western Amazon basin, especially in the northern part of Peruvian Amazon, but the distribution extends also to Brazil, Colombia, Ecuador, Venezuela, Bolivia or Guyana (Figure 6). For Peruvian populations, the main area is around rivers of upper amazon basin. In Brazil the distribution extends into the states of Pará, along the river Trombetas, Cachorro and Mapuera. Except of the Amazon basin, this shrub has been introduced into state of Florida in USA (Rodrigues 2001; Pinedo 2004; Rodrigues & Marx 2006).
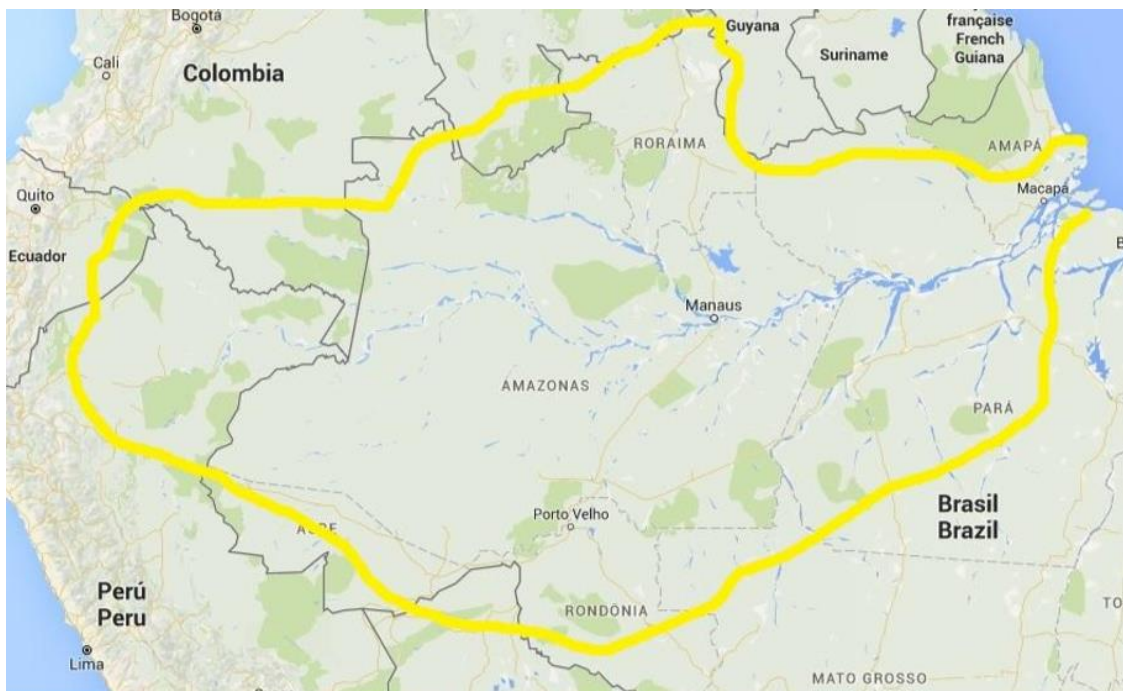
Figure 6. Approximate map of natural distribution of *Myrciaria dubia* in South America according to Rodrigues et al. 2001; Pinedo et al. 2004; Rodrigues & Marx 2006; Martin et al. 2014; Šmíd et al. 2016.

2.1.4 Cultivation and uses

Originally, camu-camu has mainly been used as a source of firewood, fish baits and shelter for the fish. The plants for cultivation were usually taken from the wild. The fruit has become popular in preparation of drinks, popsicles, candy and even cosmetics and cosequently attention

of deforestation, even supported by the government. On the other hand, the relatively easy establishment of plantation and quick profits from this plant could be a good way for local people to gain more money for personal development (Penn 2008).

2.1.5 Nutrient content and production

Camu-camu is a plant that is characterized by the production of fruit with extremely high levels of ascorbic acid and it is also considered as one of the richest sources of vitamin C. It is having about 30 times higher content of vitamin C then an orange. (Penn 2008; Rufino et al. 2010). The amount of vitamin C in the fruit pulp is varying from 845 to 7355mg/100g, depending on the stage of ripeness (Rodrigues et al. 2016b). The maximum value of ascorbic acid content was found in semi-ripe fruit (between 50-70% ripe) (Villachica 1996). The differences in content of vitamin C in the fruits among the stages of maturity may be caused by the diversity of the ecotypes and genotypes of the camu-camu, environmental factor of sourcing areas, water and soil pH, temperature, influence of nutrients to the biosynthesis of vitamin C and the amount of flooding (Rodrigues et al. 2016b).

Because of such special growing requirements of camu-camu, the possibilities for intercropping are limited.

Any specific management of the trees in not needed after final establishment of the field, on the other hand, elimination of weeds needs to be done frequently in early years in order to reduce competition and during dry season, when seedlings are young. In areas with non-floodable soil is recommended the use of cover crops (*Arachis pintoi)* (Vallachica 1996). Pruning during the growth is done rarely, usually only in case of removing dead or damaged branches. To provide better yield and condition, it is recommended to use organic fertilizers (Rodrigues et al. 2001; Pinedo et al. 2004).

Around 46 % of flowers are pollinated by small bees, that are attracted by the sweet nectar and the rest of the flowers are pollinated by wind, an average of 15 % of the immature fruits abort before reaching maturity (Andrade 1991).

Usually the first fruits appear 2-4 years after establishment, but it takes around 6 years for a full production to start. Harvest is done by manual collection of the fruits every 3-4 days. The ideal ripening stage for harvest is when the colour is changing from green to purple, at this point the fruit reaches the highest content of vitamin C (Rodrigues et al. 2001). Harvesting of the naturally occurring fruit populations in Peru is done usually from the second half of December until March and due to the water level use of canoes is necessary. The period of harvesting on plantations lasts longer, from November to May (Pinedo et al. 2004).

The maturity stage of the tree is directly correlated with diameter of the trunk. A tree is mature, as soon as it reaches approximately 12 cm in diameter, such a kind of tree is capable to produce about 30 kg of fruit per year. The yield of camu-camu in Peru is estimated between 11.7 -20.8 t/ ha/ year considering the possibilities of the farmers (Rodrigues et al. 2001; Pinedo et al. 2004).

The demand for this fruit is growing continuously, so there is also an increase in establishing new plantation to comply with the demand. This leads local farmers to exploit the wild populations for planting. However, camu-camu is a fruit consumed not just by humans, but it also is an important source of food and shelter for some species of fish (e.g. *Colossoma macropomum*) (Penn 2008). Extensive collecting fruits from natural populations caused that abundance of these species dropped down or even disappeared (Peters & Hammond 1990; Penn 2008). It is expected, that higher demand for camu-camu will be leading to an increased level

Usually, seeds are used for propagation of camu-camu, they are removed from the ripe, deep violet fruit and detached from the pulp by hand and then rinsed in cold water (FAO 1986; Picon et al. 1987; Rodrigues 2001; Villachica 1996).

After removal, the seeds are put in a well-ventilated and shady place for one hour to get rid of excess water. The preparation process needs to be done quickly, since the seeds rapidly lose their germination power. Broken and unsuitable seeds must be eliminated (Villachica 1996; Rodrigues 2001).

The seeds must be pregerminated. They are put into containers of composted sawdust, which are later placed in a suitable place for easy watering and with good airflow. Germination starts within 10 days and lasts up to 25 days (Chagas et al. 2012; Ferreir 1986; FAO 1986; Rodrigues 2001). Suitable substrates are sand or vermiculite. The seeds should stay in a shady or semi-shady place. Young seedlings up to 20 cm of height should be watered daily (Chaga et al. 2012).

Another method that can be used, is vegetative propagation by stem cuttings and grafting. These asexual methods work well for fast growth of uniform population. However, they bring the risk of infection or transferring both viruses and bacteria. For stem cuttings, the woody or semi-woody branches of young shoots are the most suitable ones and to improving rooting anility, application of phytohormones is recommended. The best results recorded are with naphthalene acetic (NAA) at the concentration of 200mg/l (Chagas et al. 2012; Suguino 2002; Galucio 2002).

When it comes to the mortality rate, grafting seems to be a better option for vegetative propagation than stem cuttings. As for the methods of grafting, cleft and whip grafting are used the most, whereas the lowest mortality (35 %) was observed in case of cleft grafting. For rootstocks, it is possible to use seedlings of other *Myrciaria* species or *Psidium guineense*, which are suitable for this purpose and grow faster than camu-camu plants (Chagas et al. 2012). After grafting, the plants should stay in the same bed from 6 to 8 months, until pruning is visible, then they are ready to be planted in the field (Clay & Climent 1993). The planting distance between plants is usually 3 or 4 m with recommendation from 833 to 1,111 plants/ha (FAO 1986; Chávez 1988; Villachica 1996).

As previously mentioned, camu-camu is cultivated as a monoculture. This technic seems promising in western Amazonia (Peters et al. 1989). However, there are some farmers who are using intercropping with cassava (*Manihot esculenta*) or water melon (*Citrullus vulgaris*).

of reaserchers started to grow during the 1950s when camu-camu entered interational trade. The biggest demand of fruit pulp had Japan (Penn 2008).

For these reasons the experimental cultivations projects began in the 1960s. From these projects was clear the potencial of camu-camu as a flood-resistant and highly productive crop. Subsequently, the production of camu-camu in Peru increased rapidly in the mid-1990s. The aims of the cultivations projects were to plant 10,000 hectars to cover the export demand of Japan. In to the production were included maily rural smallholders to raise their houshold incomes and sustainable development. However, because of its small support and hurry in development of production, only small number of farmers of camu-camu remained (Penn 2008).

This species has one big advantage for the future, and that is the fact, that it naturally grows in monoculture stands (Figure 7). Also, thanks to flood periods, the stands have less problems with pests (Clay & Clement 1993).

Figure 7. Plantation of camu-camu at the beginning of dry season (author: Ing. Jan Šmíd).

Because of its growth conditions and requirement for full sunshine, camu-camu is not an ideal candidate for cultivation in agroforestry systems (Picon et al. 1987). Unless there is an agroforestry system based on camu-camu and aquaculture (Penn 2008).

Table 1. Camu camu fruit composition on a fresh weight basis at harvest (half-mature fruit according to different authors) (modified from: Rodrigues & Marx 2006).

Component/100 g	Reference			
	Villachica (1996b)	Rodrigues (2002)	Zapata and Dufour (1993)	Institute Sinchi (2008)
Water (g)	94.4	94		
Protein (g)	0.5	0.4		0.01
total carbohydrate (g)	4.5	3.5		0.02
Fibre (g)	0.6	0.1		0.02
Ash (g)	0.2	0.3		
Lipids (g)	0.2	0.2		
Citic acid (g)			1.9	
Fructose (g)			0.95	
Glucose (g)			0.82	
pH		2.89	2.56	
Soluble solids (°Brix)		6.9	6.8	
Acidity (g citric acid)		1.8	3.8	
Vitamin C (mg)	3,253	1,410	960	2,996
Riboflavin (mg)	0.04			
Niacin (mg)	0.06			
Total amino acids (mg)			63.7	
Serine (mg)			31.6	
Valine (mg)			28.9	
Leucine (mg)			19.9	
K (mg)		83.88	71.1	
Ca (mg)	27	15.73	6.5	0.03
Mg (mg)		12.38	5.1	
Na (mg)		11.13	2.7	0.01
Fe (mg)	0.5	0.53	0.18	0.22

Apart from that, it is also a great source of phenolic compounds which give camu-camu antioxidant properties, other bioactive phytochemicals can be found in the pulp, for example flavonoids and anthocyanins (Fracassetti et al. 2013; Lopes 2015). Regarding mineral content, camu-camu is relatively limited (Table 1) only potassium stood out with content ranging from 71.1 to 83.88 mg/100g pulp, according to Zapata & Dufour (1993), Justi et al. (2000).

The fruit is usually not consumed raw, because of its acidity and bitterness. To make camu-camu tastier, it is used for preparing juices, ice creams or pastries, it can also be mixed with

cane alcohol to prepare homemade liqueur, traditionally called "camu-camuchada. Dried pulp can be added into water and drank hot or cold (Peters & Hammond 1990). It is also used in medicine as a supplement to an ordinary diet. It is considered that camu-camu is useful for treatment of herpes, headaches, migraine, gallstones and, especially, cold and severe flu (Fernández et al. 2001). For the product is used pulp, but the yield of the pulp is low only 50-55 % is its share in the fruit and the rest is representing seeds and skin (38-40 %) (Rodrigues 2001).

Until 1998, the production of camu camu was around 8t/year/tree, but the demand of the international market caused an increase in production. During the year of 1998 the demand was 20,000 t, nowadays it increased to 230,000 t /year (Rodrigues et al. 2001). The overall average yield of the wild populations varies from 8-18 t/ fruits per ha (Rodrigues et al. 2001). In case of yield from the plantations, the numbers vary from 10-14 t/ha (PROAPA-GTZ 2000).

Production of camu camu brought good earnings, when the redemption price of the fruit was between 0.5 and 2 USD/ kg in 2008. Intensive fruit extraction continued with the highest harvest in years 1997-2001 and in 2007 exports reached their peak and totaled almost 5 million USD (Pinedo et al. 2010; Martin 2014). Total area of plantations of camu camu in 2009 was 3,570 ha with production up to 13,500 t. From all production, 49 % was exported to Japan, 43 % to the USA and around 7 % to Canada, Great Britain, Germany and Australia (Pinedo et al. 2010). From 2012-2016, the export situation changed. Japan has remained main importer with 44 % of all sales. Second is Italy with 22.19 %, followed by USA with 12.1 % (Canopy Bridge 2017).

2.1.6 Pests and diseases

Camu-camu has one advantage against pests, and that is the seasonal flooding of area of natural or cultivated plantations which lowers the risk of diseases or pest. However, there are some (Figure 8 and 9). Camu-camu was influenced by numerous phytopathogenic insects with around 70 species (Delgado & Couturier 2004; Penn 2006). In the Table 2 are summarized main pests that can be found on camu-camu and which can be potentially dangerous to plantations. Pest control consists of removing the affected part of plant or biological protection, but chemical defence is not used yet (Rodrigues et al. 2001).

Figure 8. Leaves of camu camu attacked by aphids.

Figure 9. Brown spots are symptoms of a fungal infestation caused by *Pestalotia* sp.

Table 2. Pests occurring on camu-camu plants (Rodrigues et al. 2001).

Pest	Description	Damage	Control
Aphis gossypil	Homoptera, Aphidiae Pale yellow-dark green	Desiccation of shoots and leaves	natural adequate beneficial insects
Austrotachardiella sexcordata	Homoptera, Kerridae, Brown star shape	Total or partial desiccation of branches	cut and destroy affected branches
Ceroplates flosculoides	Homoptera, Coccidae,Golden	Kills badly infected shrubs	natural parasites and predators, frequent visual control
Conotrachelus dubiae	Curculinoid,Dark brown covered by light brown scales	Darkening of fruit to brown	anticipate the harvest and remove affected fruit
Dysmicoccus brevipes	Homoptera, Pseudococcidae, Females covered by white waxy secretion	Produce necrosis, disappearance of bark and tree death	Combat ants
Ecthoea quadricornis	Coleoptera	Females cut the branches after laying eggs (branches contaminated with larvae	destroy affected branches found on soil
Laemosaccus sp.	Greeny grey with two dark patches, Curculionid,Black, brown feet	Desiccation of branches	cut and destroy affected branches
Parasaissetia nigra	Homoptera,Yellow when young, becoming purplish brown	Induce reduction of photosynthesis	natural *avispita* parasites, chemical not justified
Tuthillia cognata	Homoptera, Psyllidae, Light brown	Deform young leaves limiting growth of shoots	natural: inefficient, chemical: needs to be studied
Xylosandrus compactus	Scolytidae, Female:shiny and dark, Male: light brown	Leaves dry out and stalks and branches die	Burn and destroy the plants as soon as discover first symptoms
Apioscelis bulbosa	Orthoptera, Proscopidae	Feed on leaves	-
Edessa sp.	Pentatomidae, Green	Dries out shoots	-

(Wang et al. 2009). These irregularities are used to determine the differences between species, individuals or populations (Gupta et al. 1996).

Microsatellites are so commonly used for their wide range of applications and because they are easy to amplify, clone and sequence by PCR method (Gupta et al. 1996; Larsen et al. 2009). The final products after PCR are possible to visualize by electrophoresis on polyacrylamide gel or agarose gel with ethidium bromide or for more exact results the products of PCR can be visualized by capillary electrophoresis (Gupta et al. 1996).

2.2.1 Molecular markers

Molecular markers are one of the most used markers. They are sequences of DNA bound to specific place in genome and it is easy to determine position of this sequence. This makes quite simple to observe inheritance into the next generations. Based on polymorphisms in DNA we can do factual evaluation of variability. Polymorphisms in DNA mean that chromosome which is carrying the mutant gene can be clearly determined from chromosome with normal structure. Ideal marker to work with is often an easily findable sequence within the whole genome which is possible to reproduce (Weising et al. 1995).

We differentiate dominant and codominant molecular markers. Difference between them is that with codominant marker we can recognize homozygous and heterozygotes, but with dominant is not possible, so we get less information from them (Govindaraj et al. 2015).

Another division of these markers is into two groups: one, where marker is based on hybridization, called non-PCR based markers and second are PCR-based (Rentaría 2007; Kumar et al. 2009).

PCR-based markers work on principle of amplification of DNA sequences. After this procedure they are sorted by electrophoresis to different sizes of DNA segments. PCR-based markers that are usually use are RAPD (Random Amplified Polymorphic DNA), AFLP (Amplified Fragment Length Polymorphism), ISSR (Inter Simple Sequence Repeats) or SSR markers (Microsatellites or Simple Sequence Repeat) (Kumar et al. 2009). These types of markers were developed for diversity analyses, clonal fingerprinting or for development of genetic maps. PCR-based markers allow the breeder to find genes, which are causing disease or pest resistance, color of the fruit or help to identify cultivars (Drew 1997; Arias et al. 2012).

2.2.2 Microsatellite (SSR) markers

Microsatellites or simple sequence repeats (SSR) are highly polymorphic genetic markers with dominantly inherited alleles. They consist of tandem repeats of short motifs (1-6 base pairs sequences of DNA). They are frequently and randomly dispersed in the genome of prokaryotic and eukaryotic organisms (Gupta et al. 1996). The number of nucleotides is variation in each organism and this is caused by several possibilities like, single stranded DNA slippage, double stranded DNA recombination, mismatch/double strand break repair, and retrotransposition

crossbreeding and low gene flow appeared. In these growing conditions the bottleneck effect can be one of the possible explanations for high inbreeding values.

The microsatellites used in these studies showed high levels of genetic diversity and proved to be useful for genetic analyses in populations od *M. dubia*.

Table 3. Seven primers developed for *Myrciaria dubia* in populations of plants in Peru (Šmíd et al. 2016).

Locus	Repeat motive	Ta (°C)	Size (bp)	k	Ho	He
MDI010	(CTT)12	56	95	9	0.408	0.680
MDI006	(CT)5(CT)6(CT)12(TCG)11	56	195	3	0.137	0.218
MDI015	(TC)12	56	305	8	0.421	0.626
MDI004	(CTT)10	56	397	4	0.284	0.397
MDI009	(GA)18	59	363	10	0.426	0.661
MDI007	(CTT)10	58	245	3	0.296	0.354
MDI003	(CT)6(AG)14	59	156	4	0.527	0.647
Average					0.357	0.512

Ta: annealing temperature; k: number of detected alleles per locus; Ho: observed heterozygosity; He: expected heterozygosity; bp: base pair

2.2 Genetic markers

Genetic markers are an easily identifiable piece of genetic material (usually DNA), that are used in the laboratory procedures to distinguish cells, individuals, populations, or species. For biochemical markers we extract proteins or chemicals. In case of molecular markers, we work with extracted DNA from plant tissues (seeds, leaves, etc.). Using these markers can help us to evaluate variability within and among population, differences between natural and domesticated populations, genetic structure, gene flow, hybridization and we can detect the important populations or individuals that are suitable for breeding programs or developing of new varieties (Williams et al. 1990; USDA 2006). The choosing of the right marker should be based on characteristic of the primer and on which species the research is focused on. There are a lot of possibilities. Important factor lies in whether the marker is dominant or codominant (USDA 2006).

2.1.7 Diversity of *Mycriaria dubia*

As previously mentioned, the natural distribution of *M. dubia* is along rivers, banks and lakes in Amazon basin, mostly in Peru and Brazil. Because the Amazon river basin is spreading over large plains and the distances separating individual rivers are quite large, the diversity between population could be relatively high (Rojas et al. 2011).

In Peru was observed high phenotypic diversity between populations of camu-camu, especially in terms of size and weight of fruit, amount of pulp in fruit, content of ascorbic acid, time of ripening and in germination process (Vásquez 2000). In department of Ucayali, where most of the populations are semi-captive or domesticated, the diversity is quite low. However, in Loreto department in Iquitos where the populations around rivers appear mostly naturally, the variability was the highest (Pinedo et al. 2004).

M. dubia could be a particularly important fruit on the future market, but most of the scientific studies were on the nutrition site of interest. The few studies that were focused on the theme of genetic diversity were mainly from the side of Peru and Brazil (Pinedo et al. 2004; Koshikene 2009; Rojas et al. 2008; Rojas et al. 2011).

Šmíd et al. (2016) defined genetic diversity among populations of *M. dubia* in Peruvian Amazon by using seven microsatellite markers (SSR markers) (Table 3). For the study were used samples from natural populations of camu-camu from experimental field of IIAP. The levels of genetic diversity in this study were determined as high. The expected heterozygosity was higher than observed heterozygosity and so as inbreeding coefficient had high values. Similar results were recorded by Rojas et al. (2011) and Koshikene (2009) in Brazilian populations. However, the values of expected and observed heterozygosity of wild populations obtained by Šmíd et al. (2016) were higher, then in work of Rojas et al. (2011) and Koshikene (2009). The average value of inbreeding coefficient was 0.377 and average number of alleles 12.75 were determined in Brazilian study by Koshikene (2009). Lower values of genetic diversity in the study of Šmíd et al. (2016) were explained by smaller area of sampling. The inbreeding coefficient turn out to have high values. These results could be explained by several ways. Possible explanation of high diversity is large distance between populations, so the populations are isolated from each other. Also, migration of species is less possible, because the seeds are mainly spread by water. The higher inbreeding coefficient could be caused by dense growing of small populations in oxbow lakes. Thanks to this, according to Rojas et al. (2011) the

3. OBJECTIVES

The main objective of the study was to determine the genetic diversity among and within cultivated and natural populations of camu-camu in Peruvian Amazon and to find, if there is a genotypic influence on vitamin C content in the fruit.

The specific objectives of the study were:

1) To assess the intra- and interpopulation genetic diversity of wild and cultivated populations of camu-camu in the area of Iquitos and Pucallpa by using SSR primers.
2) To determine the approximate origin of cultivated populations.
3) To determine the influence of genotype on the vitamin C content in fruits of selected populations of camu-camu.

The main hypothesis of this study was that diversity between populations could be quite high due to the isolation by Amazonian forest. It was also assumed that the genetic differences between cultivated and wild population will be small because the breeding process is still at the beginning and farmers still use wild plants and seeds for establishment of the plantations.

4. MATERIALS AND METHODS

4.1 Study area and sampling site

Peruvian Amazon has two main areas that are most important for production of camu-camu fruits: Iquitos, the capital city of Loreto department and Pucallpa, capital of Ucayali department (Figure 10). Since 1994, around 500 t of camu-camu were exported from these cities. Most of the fruits were collected from wild populations (Penn 2004).

Figure 10. Map of Peru with the study sites marked in red.

Iquitos, the capital city of Maynas province, is located in the Loreto Region in the Northeastern part of Peru, close to the borders with Colombia and Brazil. This city is located on the left bank of the Amazon River and is surrounded by rives Itaya and Nanay. It has four districts: Iquitos, Belén, Punchana y San Juan Bautista, and almost half a million inhabitants living in an area of 119,859.40 km². That makes Iquitos the sixth most populated city in the country. It is the main

Peruvian fluvial port and one of the most important ones along the Amazon. Iquitos is accessible only by plane or by boat.

Being close to the equatorial line, Iquitos has a tropical rainy climate (hot and humid), with temperatures ranging from 20 °C to 36 °C. The average annual temperature of Iquitos is 28 °C, with an average relative humidity of 85 %. The average annual rainfall is 2,616 mm and the rainy season is from November to May, with the fluvial network at its highest point in May and its lowest level in October (Figure 11) (Geographical names 2000).
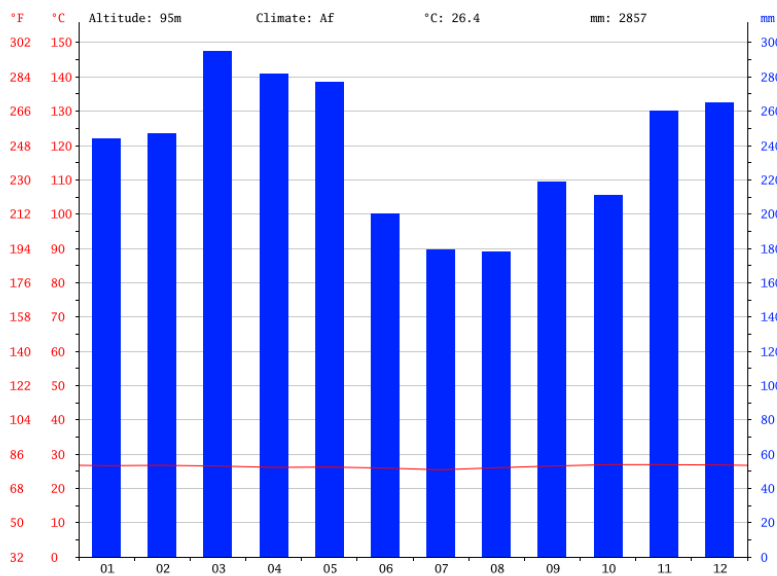
Figure 11. Climate diagram of Iquitos (Source: en.climate-data.org).

Production of Iquitos is mainly gas, petroleum, timber, oil, fruit and fishing. Regarding fruit production, camu-camu is the most important product for Iquitos, in Maynas province lies the largest camu-camu plantation in Peru counting around 2,200 ha. Another area with production of camu-camu is Loreto department with 6,500 ha of plantations (Pinedo et al. 2004).

The samples from Iquitos were collected from experimental field of the IIAP (Instituto de Investigaciones de la Amazonía Peruana) placed in village San Miguel (GPS 3° 40´ and 3° 45´ S and 73° 10´ and 73°11´ W). This village is located 10 km from Iquitos. It is an area with seasonal floods and it is surrounded by the Amazon and Itaya rivers. This field covers around 60 ha and is used for research of the IIAP and students of the UNAP (Universidad Nacional de la Amazonia Peruana).

Pucallpa is the second biggest producer and seller or camu-camu in eastern Peru, where the production takes place on the banks of Ucayali river (Pinedo et al. 2004). It is quite a young city, since it was established in 1840. Since 1945, when was the road from Lima to Pucallpa was complete, the development of the city increased. The main products of this city are timber, fruits and brewing industry (Zumaeta 2006). The most frequently cultivated crops in this area are cassava (*Manihot esculenta*), plantains (*Musa sp.*), coconut (*Cocos nucifera*), sugar cane (*Saccharum officinarum*), cocoa (*Theobroma cacao*), rice *(Oryza sativa)*, camu-camu (*Myrciaria dubia*), pineapple (*Ananas commosus*) and citruses (*Citrus ssp.*) (Zumaeta 2006).

The climate is similar to the climate in Iquitos, but there is quite a big drop of precipitation during June, July and August. The average rainfall is about 1667 mm/year and average temperature is 26°C (Figure 12).
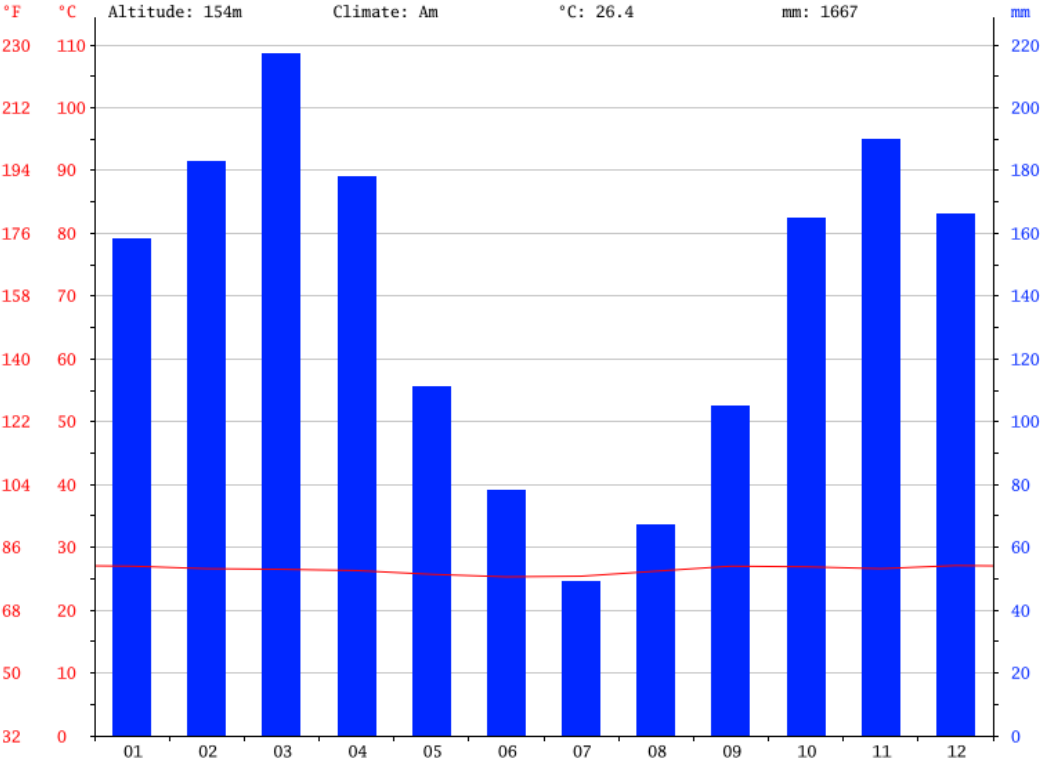
Figure 12. Climate diagram of Pucallpa (Source: en.climate-data.org).
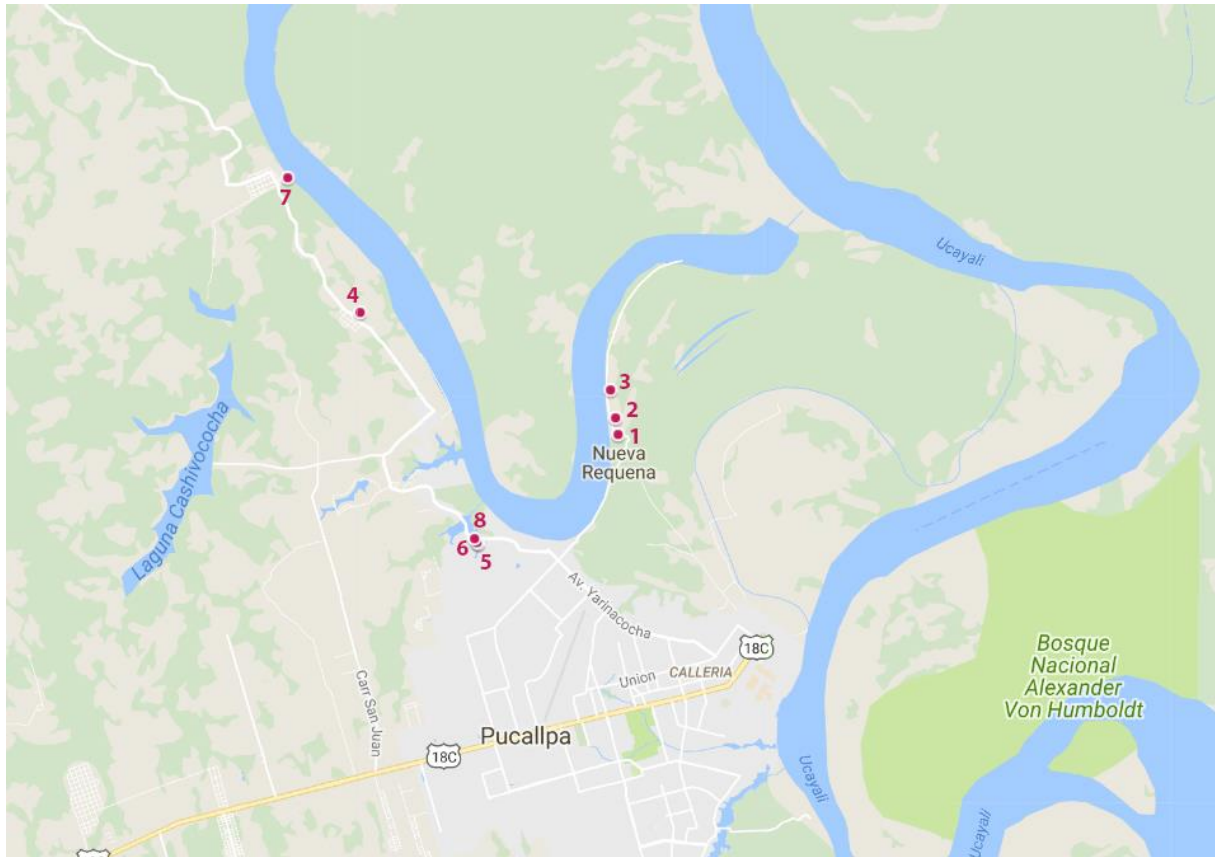
Figure 15. Cultivated populations close to Pucallpa. Populations:1-Y1; 2-Y2; 3-Y3; 4-SRS; 5-SJM; 6-FRA; 7-SFA; 8-SJP

4.3 Genetic analysis

4.3.1 DNA extraction

The extraction of DNA and PCR reactions were performed in the Laboratory of Molecular Biology of Faculty of Tropical AgriScience at the Czech University of Live Sciences in Prague and the subsequent fragmentation analyses of PCR products were done in the Laboratory of Molecular Genetics at the Faculty of Environmental Science.

The extraction of DNA was done by modified CTAB method (Doyle & Doyle 1987). As first step, the dried leaves were crushed by homogenizer. Approximately 0.1g of sample was put into tubes with glass beads and ground for 2 min at 30 cycles/sec.

Crushed sample was mixed with 500μ of CTAB buffer (2% CTAB, 1.4M NaCl, 0.1M Tris-HCl (pH 8.0), 20mM EDTA (pH 8.0), 1% PVP) and 10μ mercaptoethanol and incubated for 30 min
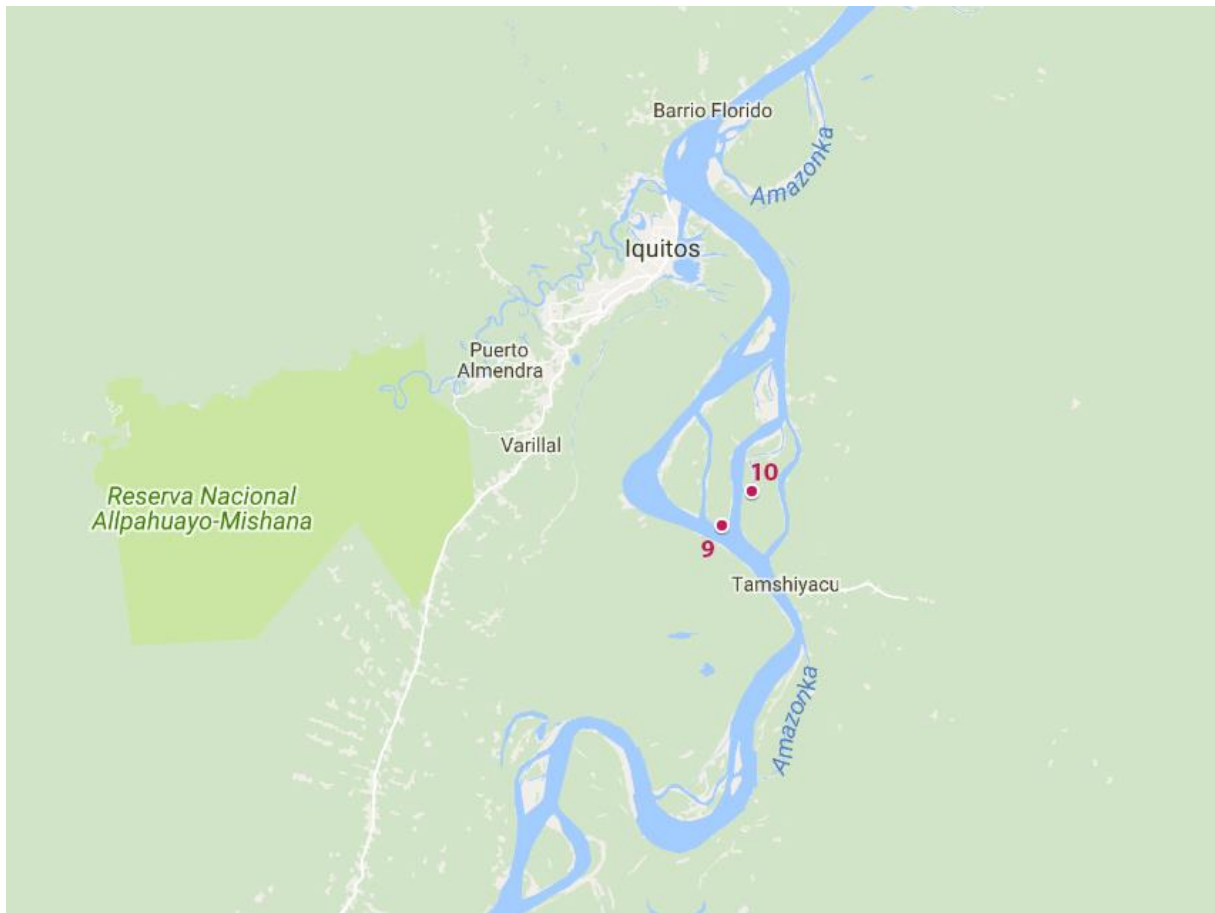
Figure 14. Cultivated populations nearby Iquitos marked in pink. Populations: 9-IG; 10-IM

Figure 13. Collection sites of chosen natural populations, transplanted by IIAP to San Miguel, marked in pink in province Loreto. Populations: 1-PM; 2-Pc; 3-PC; 4-CU; 5-CC; 6-Ct; 7-NN; 8-NY; 9-IP; 10-TH; 11-TAO; 12-THT; 13-Y5; 14-JSB; 15-YL; 16-TAHU; 17-TAAF; 18-MT; 19-MY; 20-TC MIX; 21-SUNI

4.2 Sample collection

The collection of plant samples was done during July and September 2017 in area around Iquitos and Pucallpa.

The sampling in Iquitos was performed in cooperation with IIAP on their experimental field „San Miguel" established in 2008. On this field, there is a collection of 115 diffrent populations of camu camu from Peruvian departments Loreto and Ucayali and some from Brazil. These plants are indetical to parent plants from the natural habitats. Because they were transported directly from natural habitat, we consider these population as natural and wild. From each population, 10 plants were randomly selected and transplanted. These plants further on were used for evaluation of natural populations and for selection of the best individuals and their breeding.

For our study, we randomly chose 11 populations and from each population 10-15 individuals were sampled (Figure 13). Cultivated trees were sampled also around Iquitos on 2 plantations (farms) located nearby the city (Figure 14). Another collection of cultivated plantations took place around Pucallpa on farms near villages San Francisco, Santa Rosa and San Jose. Each farm was considered as one population. From each population were sampled 10-15 plants (Figure 15). As an addition to the collected samples, data gathered by Šmíd et al (2016) were used with their consent. Results were provided from analysis of 96 individuals from 10 wild populations collected also on the experimental field of IIAP and data about 30 individuals from cultivated population located near the lake Yarina cocha, not far from city Pucallpa.

In total we have collected 385 samples of camu-camu leaf tissue, 252 from wild populations samples and 133 samples from cultivated populations (30 from Iquitos and 103 from Pucallpa), details about collected samples and populations are provided in Appendix A. These samples were stored in paper bags and then put in plastic bags with silica gel to dry. After that they was transported to the Czech Republic for analysis.

Forty representative plants from 26 different populations with the highest yields were collected for analysis of vitamin C content. All of them are growing on the experimental field „San Miguel", but they were chosen from INIA (Instituto Nacional de Innovación Agraria) and from several producers in Pucallpa. The fruits were collected in semi-mature to mature stage of ripeness, when the content of vitamin C should be at the maximum. Leaves from these individuals were also collected for analysis with SSR markers.

at 65°C while being vortexed every 10 min. After incubation were added 600μ of chloroform-isoamylalcohol (24:1) and samples were slowly mixed for 15 min at room temperature. Than the samples were centrifuged for 15 min at 9,000 RPM, the supernatant was transferred into new 1.5 ml tubes, mixed with 1ml of cold isopropanol (100%) and incubated for one hour at -18°C. Subsequently, the samples were again centrifuged for 15 min at 14,000 RPM, supernatant was discarded, and the pellet was washed with 500μ of ethanol (70%). Samples were centrifuged one more time for 15 min at 14,000 RPM and the supernatant was carefully poured out from the tube. The DNA pellet was dried at 37°C and then dissolved in 100μ ddH$_2$O and incubated overnight at room temperature. The concentration and purity of isolated DNA was measured on Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, USA).

4.3.2 SSR primers

Šmíd et al. (2016) designed 8 SSR primers for *Myrciaria dubia* from existing sequences in GenBank. After screening of all the primers, two were removed from this analysis due to lack of amplification. Final list of primers used in this study is shown in Table 8.

4.3.3 SSR analysis

Mixtures for PCR contained 5-40 ng of genomic DNA template of each sample, 1μl of both reverse and forward primers (10μM), 5μl of Multiplex PCR Kit (QIAGEN, Germany) and 2μl of PCR H$_2$O for a total volume of 10μl (Table 4) . The PCR reactions were performed in T 100 Thermal Cycler (Bio-Rad, USA). Unmarked primers (Generi Biotech, Czech Republic) were divided into two multiplexes according to their annealing temperatures stated by the company. For the first multiplex (M1: MDI004, MDI006 and MDI015) the anneling temperature was set to 51.5°C and for the second multiplex (M2: MDI010, MDI003 and MDI009) was anneling temperature 59°C. The setting of the cycler was: 95°C for 15 min, followed by 34 cycles of 95°C for 1 min, 51.5 or 59°C for 90 sec, 72°C for 1 min, followed by a hold at 72°C for 10 min (Table 5). The PCR products were subsequently displayed by electrophoresis on 1% agarose gel stained with EtBr (ethidium bromide) for visualization. This procedure was used for all samples.

Table 4. PCR composition with unlabeled primers.

Substance	Concentration	Volume Group M1
Template DNA	5-40 ng.µl-1	1 µl
Forward primer	10 mM	1 µl
Reverse primer	10 mM	1 µl
QIAGEN Multiplex PCR Kit		5 µl
water	-	2 µl
Total		10 µl

Table 5. PCR cycles for unlabeled primers.

Step	Temperature(°C)	Time (min)	Cycles
Denaturation	95	15	
Denaturation	95	1	
Annealing	51/59	1.5	34
Elongation	72	1	
Elongation	72	10	

After specifying the method, we used primers (only the forwards) with fluorescent labeling (Thermo Fisher Scientific, USA), chosen colors were: MDI003-red(PET), MDI009-green(VIC) and MDI010-blue(FAM). For primers MDI004, MDI006, and MDI0015 were in color of blue (FAM) from Generi Biotech (Czech Republic). Because the annealing temperature for labeled primers changed, we repeated the temperature gradient. The newly set temperature for M1 was 51°C and for M2 stayed the same (59°C). Final mixture of primers in modified ratio were as follows: multiplex M1: MDI006 0.3 µl (F+R), MDI015 0.6 µl (F+R) MDI004 1 µl (F+R) and multiplex M2: MDI010 0.1 µl (forward+reverse), MDI009 0.6 µl (F+R) and MDI003 0.4 µl (F + R).The solution and steps in PRC reaction remained unchanged (Tables 6 and 7).

To determine the specific length of alles, PCR products were run on capilary electrophoresis on 3500 Genetic Analyzer (Applied Biosystems, USA). The results were processed in GeneMarker® software with size standard GMC-GT 500-LIZ.

Table 6. PCR composition of all primers. Volume for multiplexes M1 and M2.

Substance	Concentration	M1	M2
Template DNA	5-40 ng.µl-1	1 µl	1 µl
Forward primer	10 mM	0.3+0.6+1	0.1+0.6+0.4
Reverse primer	10 mM	0.3+0.6+1	0.1+0.6+0.4
QIAGEN Multiplex PCR Kit		5 µl	5 µl
H$_2$O	-	1.8 µl	0.2 µl
Total		10 µl	10 µl

M1 is composed of primers MDI006, MDI015 and MDI004 and M2 is for primers MDI010, MDI009, MDI003

Table 7. Profile of PCR for labeled primers.

Step	Temperature(°C)	Time (min)	Cycles
Initial denaturation	95	15	
Denaturation	95	1	
Annealing	51/59	1.5	34
Elongation	72	1	
Final elongation	72	10	

Table 8. Microsatellite primers used in this study.

Microsatellite	5´Forward primer	3´Reverse primer	Ta (°C)	Size (bp)	Motif	Labeling
MDI003	GCATAAATAACCCCGCGGTCTC	GTACAGCTCCCAGCAGGAGT	59	160-168	$(CT)_6(AG)_{14}$	PET
MDI004	GCCTTCCAGACCCTTTTGC	GTTCTTGAACCGGGACGC	51	380-404	$(CTT)_{10}$	FAM
MDI006	GCTCTCTCTCTGAGTACCTGAAAC	CTTTCACGCAAGACCGACG	51	183-210	$(CT)_5(CT)_6(CT)_{12}(TCG)_{11}$	FAM
MDI009	CGAAGTCCTGACCTGTTCTGAGTT	GCAGACCAGCGAGTTTACACC	59	353-374	$(GA)_{18}$	VIC
MDI010	CGATCGCTGCCCTTTCTG	GGTTCGGGAGGGTAGGAG	59	88-117	$(CTT)_{12}$	FAM
MDI015	CTGTACCTGCATCGATGGTG	CGTTCTAATCCGCCATTATTCGTC	51	305-325	$(TC)_{12}$	FAM

5. RESULTS

5.1 Genetic diversity

In total we collected 385 samples from 31 populations. These samples were analysed by using seven polymorphic SSR primers. All microsatellite loci were polymorphic and 73 alleles were identified (Table 9). The highest number of alleles per locus had primer MDI009 (24) and the average number of alleles per locus was 12.17. Observed heterozygosity per locus (Ho) ranged from 0.31 to 0.96 with an average 0.49. Expected heterozygosity (He) range was from 0.41 to 0.74 with an average 0.58. Primer MDI010 displayed the highest expected heterozygosity within populations. The average of the expected heterozygosity in total population over loci (Ht) was 0.70. Inbreeding coefficient varied from -0.12 to 0.46 with an average of 0.16. The value of allelic richness ranged from 2.20 to 3.90 with MDI009 showing the highest value.

Table 9. Main measures of genetic diversity for all six loci.

Locus	k	Ho	He	HW	Ht	Fis	Allelic richness
MDI006	4	0.4632	0.4123	0	0.5013	-0.1233	2.20
MDI015	10	0.4199	0.5781	0	0.7270	0.2737	3.40
MDI004	11	0.3138	0.4644	0	0.5613	0.3243	2.57
MDI010	15	0.9554	0.7407	0	0.7761	-0.2898	3.32
MDI009	24	0.4347	0.6654	0	0.8340	0.3467	3.90
MDI003	9	0.3295	0.6062	0	0.7862	0.4564	3.37
Average		0.49	0.58		0.70	0.16	

k: number of detected alleles at the locus, Ho: observed heterozygosity, He: expected heterozygosity, HW: significance of deviation from Hardy-Weinberg equilibrium, Ht: the expected heterozygosity in total population over loci, Fis: inbreeding coefficient

The comparison of wild and cultivated populations can be found in Table 10. In case of allelic richness, the difference was quite low, but on the other hand the observed heterozygosity was higher in cultivated populations than the wild ones with. The most significant difference is in inbreeding coefficient which is lower in cultivated population.

For finding out if the genetic diversity has an effect on content of vitamin C, we used an analysis AMOVA (Analysis of molecular variance) in GenAlEx 6.51b1(Smouse et al. 2017). The variance among and within populations as percentage of the total variance was calculated with 999 permutations.

R program were used several packages. The packages poppr (Kamavar et al. 2014), hierfstat (Goudent & Jombart 2015) and adegenet (Jombart 2008) were used for calculating observed heterozygosity, expected heterozygosity, number of alleles, allelic richness, inbreeding coefficient and Hardy-Weinberg equilibrium.

The expected heterozygosity (also called Nei's genetic diversity), was calculated according to the formula:

$$HExp = \frac{2N}{2N-1}(1 - \sum_{i=1}^{l} P_j^2)$$

where Pj is the frequency for the j allele at l locus with l alleles in a population. N is the number of individuals, assuming that the populations were in Hardy-Weinberg equilibrium (Nei 1987). Inbreeding coefficient was calculated according to the formula:

$$Fis = (He - Ho) / He$$

where He stands for heterozygosity and Ho for observed heterozygosity (Li & Horvitz 1953).

The program STRUCTURE was used to divide sampled populations into several clusters. The parameters setting was: length of burning period: 10,000; number of MCMC repetitions after burning: 100,000; number of interactions: 10, also we set to use admixture model and allele frequencies to be correlated. The results were displayed using the structure harvester (Dent et al. 2009) from which determined the value Δ K. For the chosen K was done the annealing of the results from all 10 repetitions and merged by clumper 1.1.2 (Jakobsson & Rosenberg 2007). The visualization of final results was done by distruct 1.1. Data has been converted into a graphical form and inserted into map for better understanding of geographical distribution of populations (Figure 18, 19 and 20). As last step was by R package ape (Paradis et al. 2004) created neighbor joining tree based on Euclidean distances.

4.5.2 Analysis of vitamin C content

By using the STRUCTURE 2.3.4 (Pritchard et al. 2000) the data were divided into clusters based on calculating the value Δ K (Evanno et al. 2005).

4.4 Content of vitamin C

The analysis of ascorbic acid content in fruit of camu-camu was done by using modified Tillman's Volumetric method (A.O.A.C 1984).

As a first step was done a standardization, where 1ml of ascorbic acid solution was mixed with 5ml of metaphosphoric acid solution and shook vigorously. Then by titration was slowly added 2,6-dichlorophenolindophenol (DCIP), which reduced to a colorless solution and the excess unreduced dye turned to pink rose color and persist at least 15 seconds.

The formula for calculation was:

$$A = \frac{0.5}{b}$$

where the A is a coloring factor and B is a volume of DCIP used in the reaction.

After the optimization was done, we proceeded with fruit samples. Firstly, we took 10 ml of the sample and diluted to 100 ml with 3% metaphosphoric acid solution and centrifuged and then filtered to remove all solids to create the sample extract. Then we took 1ml of the extract and by titration was slowly added DCIP until the color changed to pink and noted the volume of DCIP used for titration. Lastly, we determined the vitamin C in the sample according to this formula:

$$\text{"}vitamin\ C\text{"} = \frac{A * 100}{B * C}$$

where the A is volume in ml of 2,6 dichlorophenolindophenol solution used for reaction, B weight of the fruit sample and C correction factor for 2,6-dichlorophenolindophenol.

4.5 Data analysis

4.5.1 Analysis of genetic data

For analysis of genetic data was first used program R (R Core Team 2013), free software for statistical computing and graphics, followed by STRUCTURE 2.3.4 (Pritchard et al. 2000). In

Table 10. Measures of genetic diversity between wild and cultivated populations.

Population	Allelic richness	Ho	He	Fis
Wild				
PM(19)	2.828	0.414	0.521	0.137
Pc(20)	3.109	0.321	0.485	0.291
PC(21)	3.142	0.350	0.508	0.204
CU(22)	2.907	0.417	0.487	0.154
CC(23)	3.109	0.307	0.433	0.234
Ct(24)	3.214	0.511	0.557	0.083
NN(25)	3.103	0.324	0.544	0.343
NY(26)	3.618	0.389	0.566	0.262
IP(27)	3.193	0.383	0.541	0.091
TH(28)	2.960	0.304	0.450	0.169
TAO(16)	3.146	0.342	0.571	0.363
THT(18)	2.874	0.300	0.520	0.375
Y5(8)	2.451	0.336	0.438	0.197
JSB(1)	3.481	0.400	0.616	0.251
YL(5)	3.100	0.489	0.527	0.048
TAHU(15)	2.991	0.379	0.513	0.188
TAAF(14)	3.694	0.411	0.604	0.270
MT(2)	3.200	0.303	0.479	0.223
MY(12)	4.153	0.587	0.663	0.124
TC MIX(17)	2.921	0.211	0.551	0.436
SUNI(4)	2.367	0.289	0.390	0.127
Average	3. 122	0.370	0.522	0.218
Cultivated				
Y1(29)	2.470	0.35	0.412	0.089
Y2(30)	2.979	0.269	0.507	0.371
Y3(31)	3.205	0.375	0.456	0.186
SRS(10)	2.585	0.456	0.436	-0.079
SJM(9)	3.700	0.583	0.635	0.035
?(11)	3.518	0.594	0.584	-0.078
SFA(3)	2.493	0.456	0.420	-0.113
SJP(13)	3.626	0.556	0.560	-0.008
IG(6)	3.728	0.467	0.594	0.184
IM(7)	3.602	0.470	0.644	0.120
Average	3.190	0.458	0.525	0.071

Ho: observed heterozygosity, He: expected heterozygosity, Fis: inbreeding coefficient

For calculation of the ideal number of cluster we used the STRUCTURE software and based on analysis of delta K by the Evanno et al. (2005) method was made division into three clusters (Figure16).
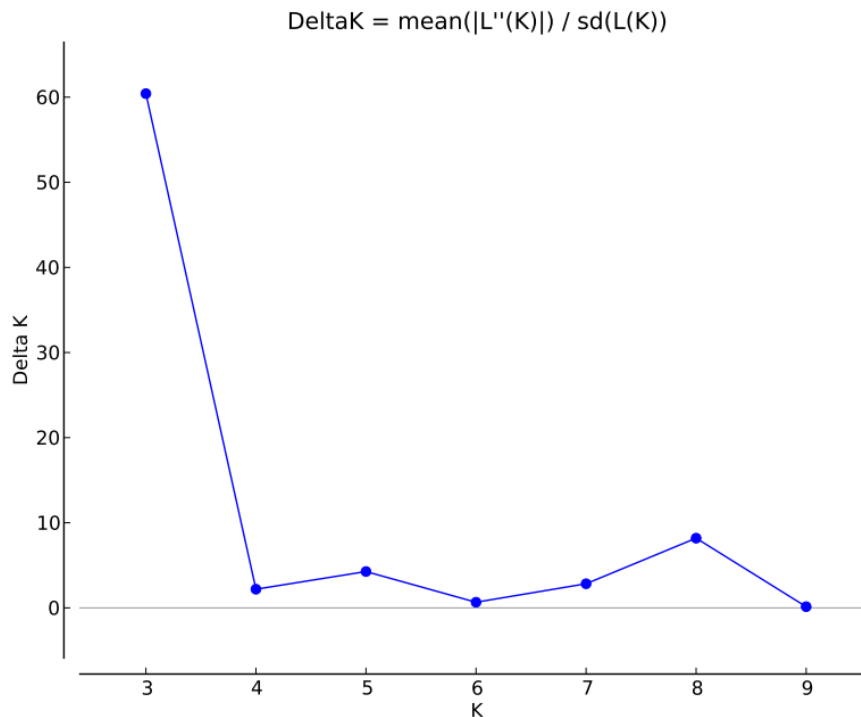

Figure 16. Delta K graph. K3 is significantly different from the others.

Based on this division (Figure 17) is possible to see that populations presented by red colour are from rivers Tigre (16-TAO; 18-THT), Tambor (4-SUNI; 17-TC MIX) and Tahuayo (14-TAAF; 15-TAHU). By burgundy colour is presented mostly river Yavari(1-JSB; 5-YL; 8-Y5) with some populations from Putumayo river (20-Pc), Curararay river (22-CU) and also the cultivated population from Yarinacocha (23-Y3) is closely related. For the pink colour, most of the cultivated population around Pucallpa fall into this group (3-SFA; 9-SJM; 10-SRS; 11-FER; 13-SJP, 29-Y3) and one from the cultivated nearby Iquitos (7-IG), but not just cultivated population belong to this group. Also, rivers Mazan (2-MT; 12-MY), Putumayo (19-PM; 21-PC), Curaray (44-Ct) and Napo (25-NN) are close to the cultivated ones. In the last group we find fewer or more mixture of the 3 divisions in wild population (23-CC; 26-NY; 27-IP; 28-TH) so as in populations cultivated (6-IG, 30-Y2). The abbreviations and the exact geographical positions of all populations are clarified in Appendix A.
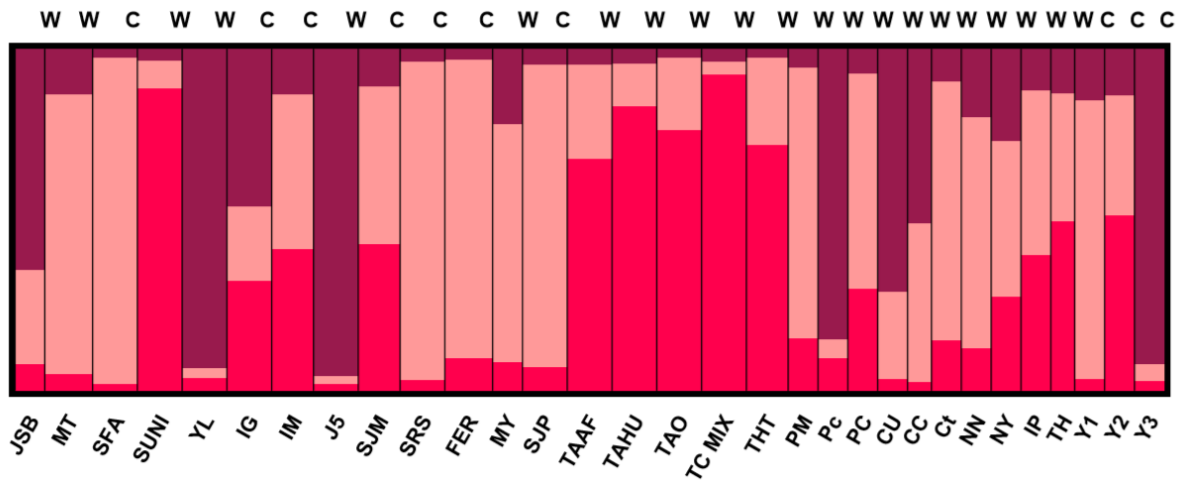
Figure 17. Division of all the populations into three clusters (W-wild population, C-cultivated population).

In the Figures 18, 19 and 20 can be seen the division into three clusters that most of the populations have mixed genotypes, on the other hand, there are some population that are almost pure and only one genotype prevails (5-YL, 8-Y5, 4-SUNI, 17-TC MIX and one cultivated population 31-Y3).
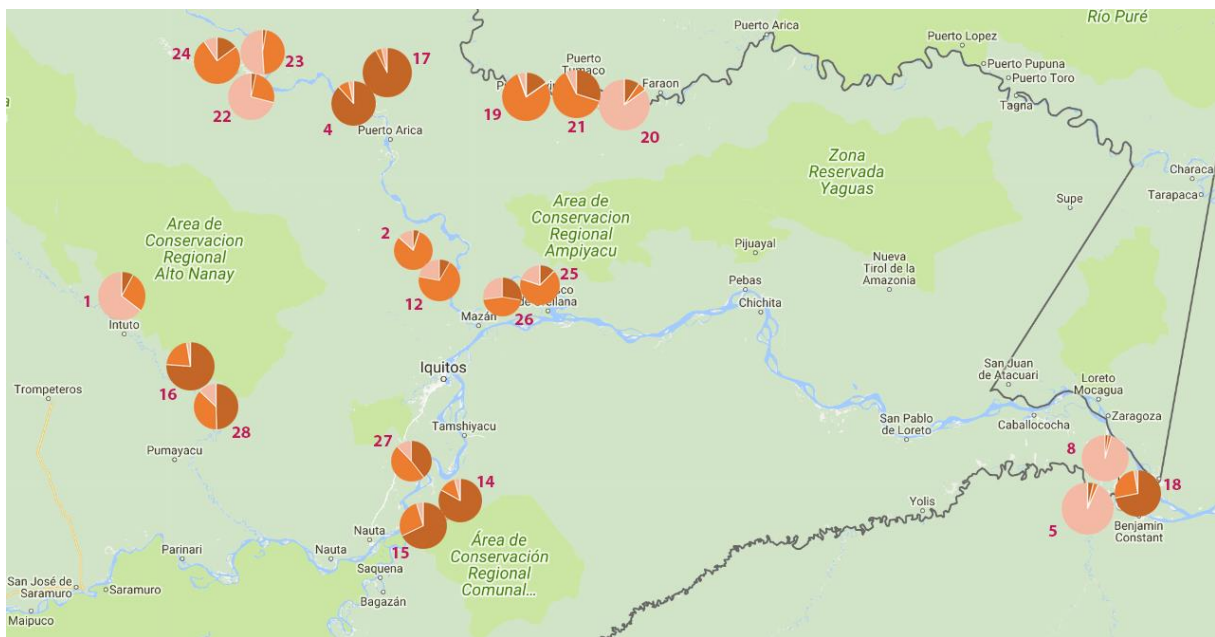
Figure 18. Graphical representation of wild populations with division into three clusters (1-JSB, 2-MT, 4-SUNI, 5-YL, 8-Y5, 12-MY,14-TAAF, 15-TAHU, 16-TAO, 17-TC MIX, 18-THT, 19-PM, 20-Pc, 21-PC, 22-CU, 23-CC, 24-Ct, 25-NN, 26-NY, 27-IP, 28-TH).

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Figure 19. Graphical representation of cultivated population nearby city Iquitos and with division into three clusters (6- IG, 7-IM).

Figure 20. Graphical representation of cultivated population nearby city Pucallpa and with division into three clusters (3-SFA, 9-SJM, 10-SRS, 11-FRA, 13-SJP, 29-Y1, 30-Y2, 31-Y3).

6. Discussion
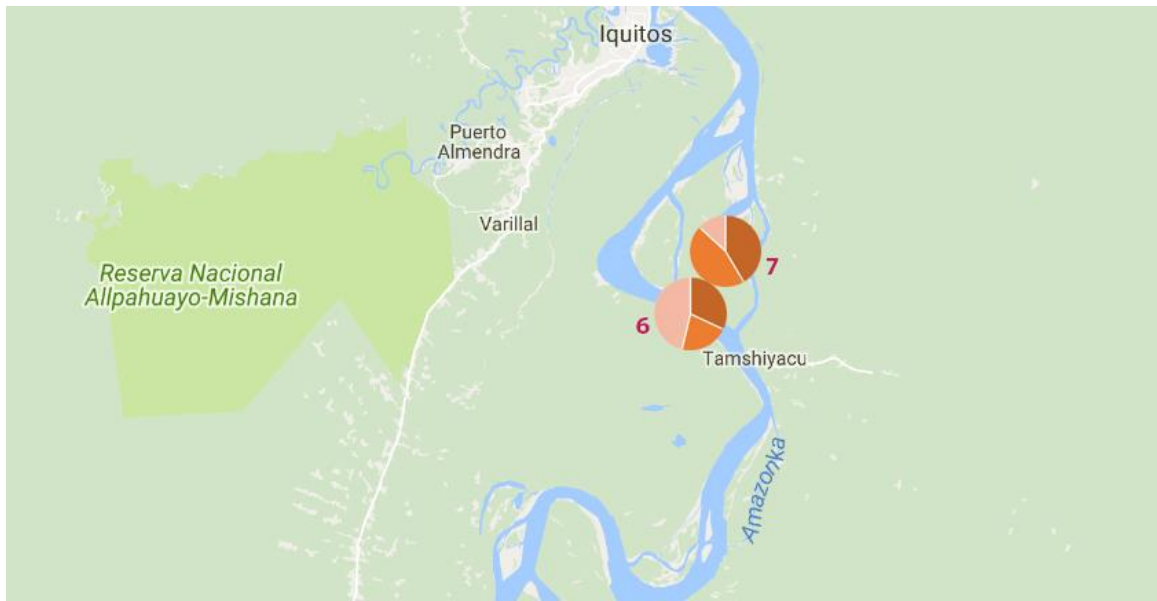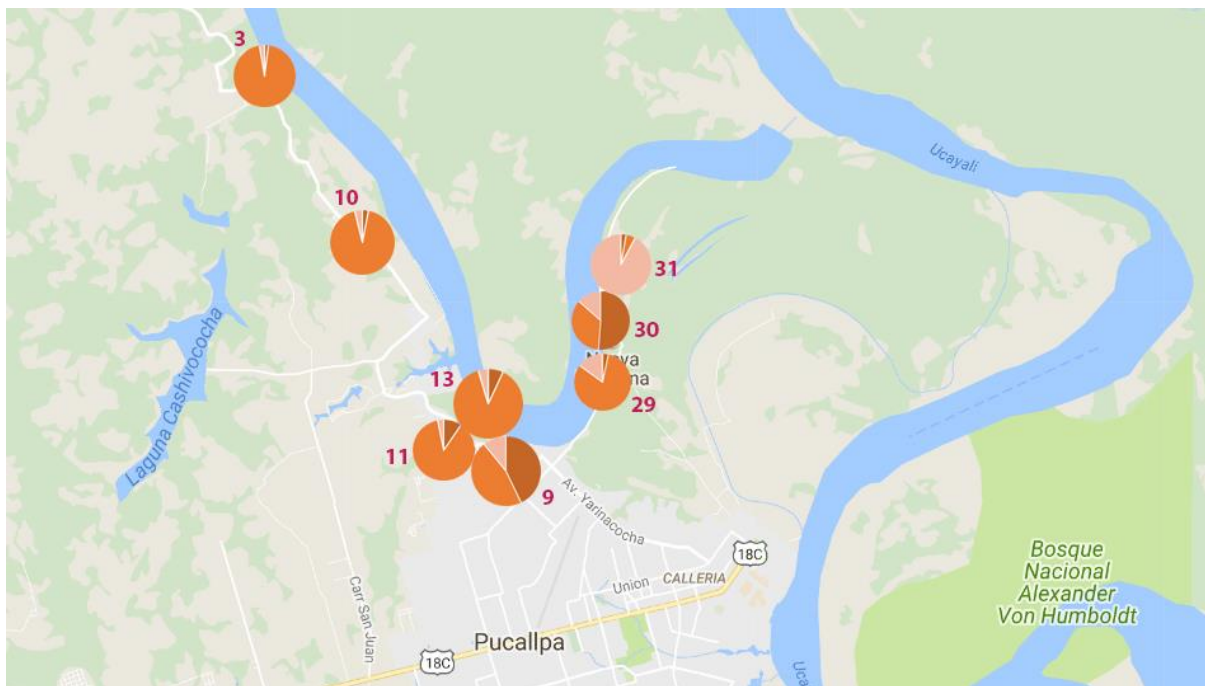
6.1 Genetic diversity

The levels of genetic diversity in our study were quite high. By using the microsatellite primers was found that expected heterozygosity was higher than observed heterozygosity, that means that the discrepancy can be caused by forces such as inbreeding. The inbreeding coefficient was quite low compared to other studies (Šmíd et al. 2016; Rojas et al. 2011; Koshikene 2009). These studies obtained comparable results to our study. In all three studies, the expected and observed heterozygosity of wild populations were slightly lower than showed our research. According to Rojas et al. (2011), who estimated the diversity of camu camu trees from different rivers in the Brazilian Amazon region by using the molecular markers EST-SSR, the average value of expected heterozygosity was 0.797 and observed heterozygosity 0.409. The average value of inbreeding coefficient was 0.377 and average number of alleles 12.75, which is mostly comparable with the results of our study except for the inbreeding coefficient, which was lower in our study. The high inbreeding coefficient Rojas et al. (2011) explained by the significant biparental endogamy detected and by human selection, given the abundance of relatives in a small area, also by the practice of planting half siblings. Koshikene (2009) reported only slightly different values compared to Rojas et al. (2011) in Brazil.

We obtained data from 31 different populations that originated from a wide range of locations. This could be a reason for lower inbreeding coefficient than in other studies than in other studies. The populations due to the distance were isolated from each other and the mixing of genes was impossible. This gave us the high value of genetic diversity. However, we can observe similarity of populations located on or near the same stem flow. Migration of this tree through the forest is less probable, but we can notice that there are some cases of populations distanced from the rivers and lakes (Figure 19). These populations could have been created by the seeds that were transferred during rainy season when the water level increases and reaches further into the forest.

The cultivated populations around Iquitos are genetically related to the wild populations from the surroundings of the Amazon river. The similarity can be caused by the origin of these populations, because traditionally the trees for plantations were taken from the wild. This explanation should be taken with caution, considering that we sampled only two populations in this area.

5.2 Vitamin C

We divided samples into five groups according to content of vitamin C (60-499, 500-999, 1000-1499, 1500-1899, 1900-2700 mg/100 g of pulp) and used AMOVA to calculate the percentage of variance among and within population of the total molecular variance. The variance within populations was 99% and among populations only 1% (Figure 22).

Percentages of Molecular Variance

Among populations 1%

Within populations 99%
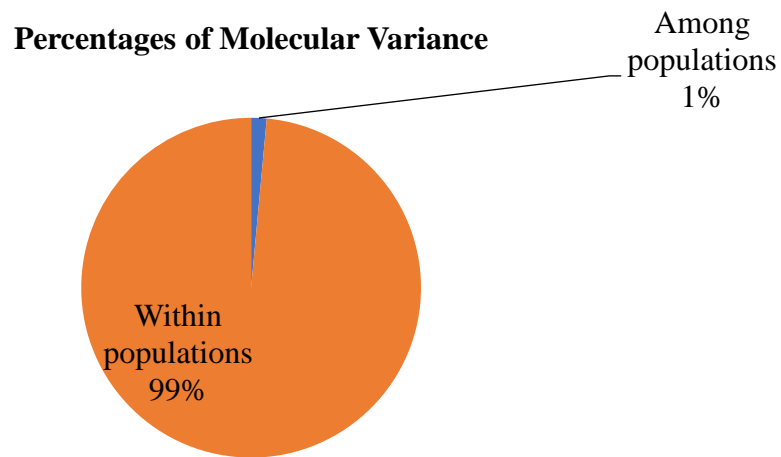
Stat	Value	P
Fst	0,015	0,189

Figure 22. A graph of variance among and within populations as percentage of the total variance.
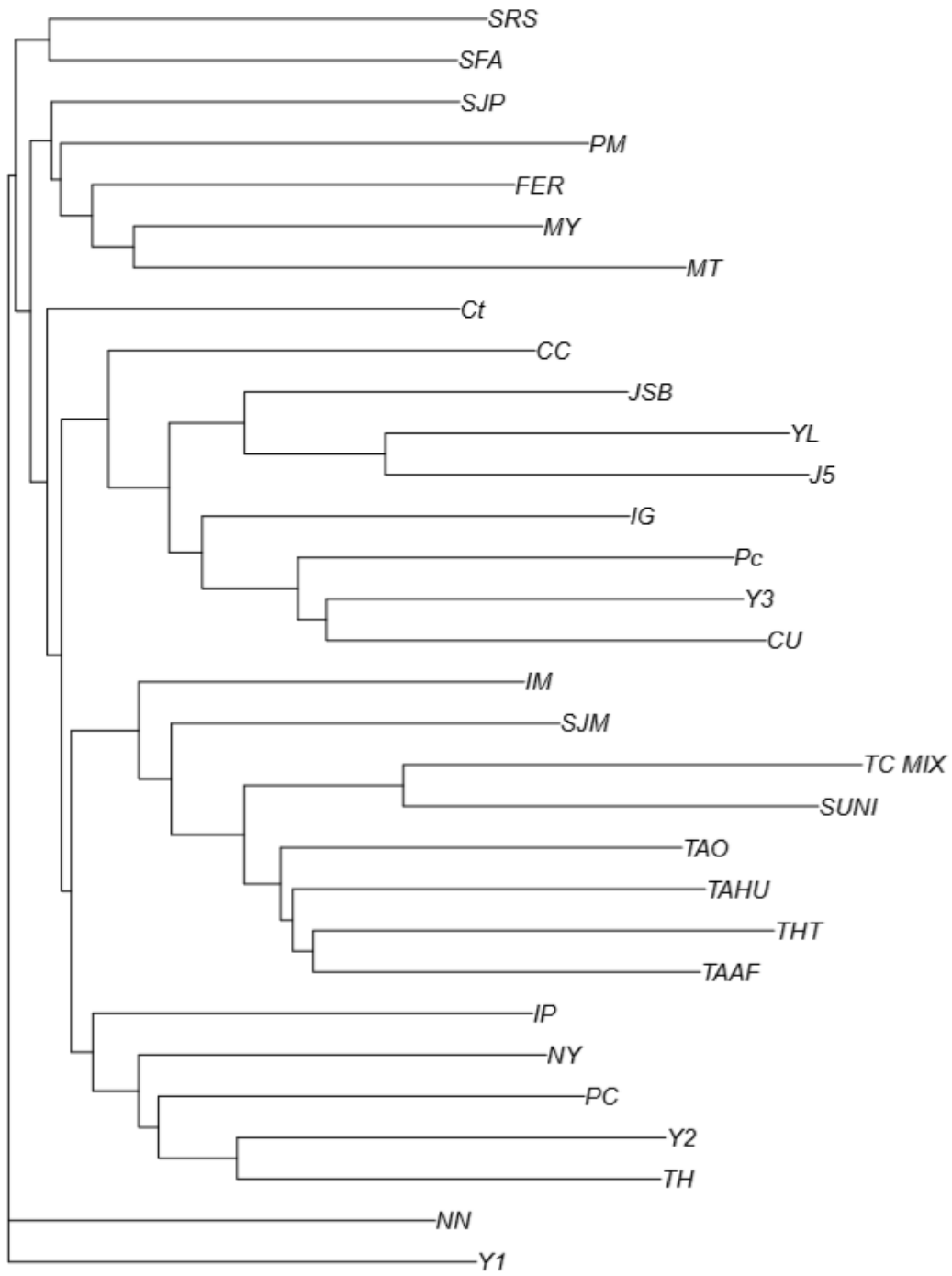
Figure 21. Neighbor joining tree based on Euclidean distances.

The results divided our populations into several groups. We can see from the neighbor joining tree that there are several possible origins, but when we focus on the dividing cultivated populations by this program we can observe interesting relations with wild populations. According to division to the cluster SJP (13) and FER (11) could be originating from rivers Mazan or Putumayo. The population Y3 in the same division as population CU (22) which is on the Curaray river and so we can assume that the Y3 (31) was taken from area of this river, also the IG (6) population should be related. In case of SJM (9) and IM (7) is possible to see connection to three main rivers Tambor (SUNI, TC MIX), Tigre (TAO, THT) and Tahuayo (TAHU, TAAF). Population Y2(30) is directly connected to wild population TH (28) located on river Tigre. SFA (3) and SRS (10) reacting individual group only cultivated population and lastly Y1(29) is belonging into totally independent group from the others (Figure 21).

The last site of sampling was Pucallpa which is nowadays one of the biggest producers of camu camu in Peru, with a lot of small populations around the city. Based on the graphical display in the map we can assumed that they are similar. As the plantations are planted close to each other with dense stands and usually low number of individuals, crossbreeding and low gene flow can occure.

It was also revealed that the populations on the border with Columbia and Brazil have different genotypes which can bring some beneficial properties to future breeding process and for this reason the most promising genotypes should be selected and preserved in germplasm banks.

In the neighbour joining tree based on Euclidean distances, it can be seen that populations are very mixed, but as we said before by looking at the results of STRUCTURE the populations located on the same river or or downstream from each other are influencing other populations by spreading the seeds by water. Looking at the cultivated populations we can assume that some of them were taken from the wild. For example, cultivated population Y3 according to the Euclidean distance should be quite interconnected with the CU population originating from the Curaray river or population Y2 with the TH population from river Tigre.

Although this study was a result of the combination of both our data and the data of Šmíd et al. (2016), it still represents a fraction of the knowledge that can be gained about this species, and it would be beneficial if this research were to continue, especially when optimized primers tested by two studies are available.

6.2 Vitamin C

In our study, we used AMOVA to find the possible link between genotype and the content of vitamin C. Previous studies confirmed the influence of environmental factors on the content of vitamin C in fruits (Chowhan 2016; Bassi et al. 2018). Specifically, for camu camu, Rodrigues et al. (2016a) discovered that the composition and conditions of soil such as flooding or fertilization by nitrogen has an impact on the biosynthesis of vitamin C. Since the trees sampled in our study are planted in one location – San Miguel, experimental field of IIAP – the ecological conditions for all trees were identical and the influence of environmental factors was excluded. The results however showed that there was almost no influence of variance among the populations. Several authors mentioned that the stage of maturity plays a significant role in the content of vitamin C in camu camu fruit (Pinedo 2012; Iman et al. 2011; Teixeira 2004; Yuyama 2002). Due to the lack of fruits during our sampling in Peru we used data about the

vitamin C content analysed by IIAP and the possible explanation of these results could be that the fruits which were collected were not in the same maturity phase and thus had different levels of vitamin C content. Several investigations revealed the influence of genotype on vitamin C in other fruit (Chowhan 2016; Rombaldi et al. 2016; Figueroa-Cares 2018; Bassi et al. 2018), however, it is possible that the six microsatellites markers used in this study are not enough to reveal this kind of influence in camu-camu.

A possible way to improve this method would be to analyse several populations with different genotypes and to collect from each individual at least one kg of fruit. Also, it is important to have minimum of three plants of each genotype for correct evaluation. The yields and content of the compounds in the fruit change rapidly, thus long-term investigation would be the most accurate. The actual content of ascorbic acid is possible to determine by other procedures than titration, e.g. as was used in study of Rombaldi et al. (2016) by high performance liquid chromatography (HPLC) using a reverse phase column CLC-ODS (4.6 mm x 150 mm x 5 μm), with a UV detector. It is important for research of influence by genotype to have the same environmental conditions for all studied plants, because it is known that environmental factors have an impact on biosynthesis of vitamin C (Rodrigues et al. 2016b; Marti et al. 2018).

7. Conclusion

In this study were used six SSR primers for evaluation of genetic diversity among and within 21 wild and 10 cultivated populations of *Myrciaria dubia* in Peruvian Amazon. All of the primers were polymorphic and can be recommended for further genetic studies dealing with this species. The average expected heterozygosity was 0.53 which is higher value than observed heterozygosity with average 0.49. The inbreeding coefficient was 0.16 and the average number of alleles per locus was 12.17 and the allelic richness ranged from 2.20 to 3.90.

The data analysis divided the wild populations into three main groups and we were able to determine the approximate origin of cultivated populations.

The approximate origin of cultivated populations was quite difficult to estimate since there was mixing of the genes, that was showed in Figure 19. However, we can say that genetic diversity was higher, when the populations were separated from each other.

Our study was also focused on the influence of genotype on the vitamin C content in fruits of selected populations of camu-camu. The molecular variance within populations was 99% and among populations only 1%. Such results show almost no effect of genotype the content of vitamin C. However, we suggest modifying the procedures to get more accurate results.

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9. APPENDICES

APPENDIX A. GPS coordinates of collected populations

Populations "Wild"	Number of samples	Code	Latitude	Longitude
Putumayo- Molano	10	PM	-2.427938	-72.786655
Putumayo- Coto	10	Pc	-2.46791	-72.520972
Putumayo- Cedro	10	PC	-2.400826	-72.627953
Curaray- Urcos	10	CU	-2.346033	-74.150949
Curaray- Chavarréa	8	CC	-2.348431	-74.123527
Curraray- Tostado	10	Ct	-2.303415	-74.228746
Napo- Núñez	10	NN	-3.36517	-72.828721
Napo- Yuracyacu	10	NY	-3.366996	-72.992825
Itaya- Pelejo	10	IP	-4.104278	-73.390674
Tigre- Huacamayo	8	TH	-3.840597	-74.37356
Tigre- Aguarume	15	TAO	-3.795801	-74.359457
Tigre- Huarmi tipishca	15	THT	-3.4353277606	-74.7855805555
Yavari- Sacambu	15	Y5	-4.253299	-70.125089
Yavari- Sacambuzin	10	JSB	-4.241317	-70.125126
Yavari- Letimana	15	YL	-4.317722	-70.118776
Tahuayo- Huiririma	15	TAHU	-4.36296666666667	-73,2516
Tahuayo- Afasi	15	TAAF	-4.3555	-73.2572666666667
Mazan- Tigre	16	MT	-3.1908055391	-73.4293611113
Mazan- Yacate	10	MY TC	-3.2851944242	-73.3451666665
Tambor- Copalillo	15	MIX	-2.3897499854	-73.5791944447
Tambor- Suni	15	SUNI	-2.4210277611	-73.5991666669
Populations "Cultivated"				
Yarina cocha 1	10	Y1	-8.328365	-74.562932
Yarina cocha 2	10	Y2	-8.324925	-74.563511
Yarina cocha 3	10	Y3	-8.318810	-74.564756
Pucallpa- SINUIRE-Santa Rosa	15	SRS	-8.302358	-74.618905
Pucallpa- MELENDES- San Jose	14	SJM	-8.351826	-74.593329
Pucallpa-unkown owner- San Jose	16	?	-8.351467	-74.593417
Pucallpa-AUGUSTIN-San Frencico	15	SFA	-8.273554	-74.634446
Pucallpa- PINCHIN-San Jose	15	SJP	-8.350528	-74.594026
Iquitos-GOMEZ-Nuevo Progreso Amazonas	15	IG	-3.9565438788	-73.2067215237
Iquitos- MARUJA- San Francisco de Angamos	14	IM	-3.9303347999	-73.1840262827