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Molecular phylogeny of *Cinchona* spp. in Peru and DR Congo

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Abstract

The national tree of Peru and Ecuador belongs to the genus *Cinchona* and is endemic to the slopes of the Andean mountains. The world's first antimalarial drug, **quinine**, was extracted from the bark of this tree, a discovery that has influenced political and economic relations through time. The genus has suffered from historical bark harvesting for extraction of antimalarial compounds and high rates of deforestation. Remaining *Cinchona* populations are predominantly found in severely degraded habitats on the edges of pastures, while a minority are protected in private reserves. Moreover, there is a lot of discussion between botanists over the identification of *Cinchona* species due to difficulties regarding species differentiation based on morphological characteristics and the confusing nomenclature within the genus.

We aimed to identify the sampled Peruvian specimens, assess a phylogenetic signal in the alkaloid production and reveal phylogenetic patterns in *Cinchona* spp. across a broad geographic area in its region of origin, Peru, where we expected high genetic variability among populations. By assessing the genetic diversity of each sampled population, we tried to pinpoint priority *Cinchona* populations for conservation. However, the number of sampled specimens was limited and our data were not ideal for population genetic inference. In addition, we aimed to identify the cultivated species in plantations in South Kivu, DR Congo, determine their region of origin, and assess the genetic diversity between plantations. Genetic profiles of leaf samples were generated using three plastid markers and one nuclear marker.

We identified the populations of Jaén and Bongara in Peru on species level as *Cinchona pubescens*, whereas the Peruvian populations from San Ramón and Rodríguez de Mendoza belong to the genus *Cinchona* but were not identified as *C. pubescens*. Populations in Bongara and Rodríguez de Mendoza deserve the highest conservation priority because these populations contained the highest genetic diversity. However, each studied population should be protected from further deforestation and agricultural expansion given the few remaining tree individuals in many parts of Peru. Cultivated accessions from DR Congo were identified as *C. calisaya*. Our results suggest that these accessions characterize the populations in the species' native range in Bolivia. However, no *C. calisaya* accessions were sampled in Peru. To delineate the region of origin of the cultivated *C. calisaya* more narrowly, a wider sampling of its full native range is needed in Peru and Bolivia. Furthermore, the cultivated genotype in DR Congo equals the variety of *C. calisaya* with the lowest quinine yield in its native range in Bolivia. However, the influence of the genotype and the environment on the quinine concentration in *Cinchona* trees remains unknown. This phylogenetic study can be used as a strong basis for the assessment of phylogenetic patterns of alkaloid production in *Cinchona*.

Samenvatting

De nationale boom van Peru en Ecuador behoort tot het geslacht *Cinchona* en is endemisch voor de hellende regio's in het Andesgebergte. Het eerste antimalariamiddel ter wereld, **kinine**, werd gewonnen uit de schors van de *Cinchona* boom. Overexploitatie van het genus omwille van zijn medicinale eigenschappen en de hoge ontbossingspercentages hebben het aantal *Cinchona* bomen sterk gereduceerd. De resterende *Cinchona* populaties worden voornamelijk aangetroffen in ernstig aangetaste habitats, terwijl een minderheid wordt beschermd in natuurreservaten. Bovendien is er veel discussie tussen botanici over de identificatie van *Cinchona* species vanwege moeilijkheden met betrekking tot soortdifferentiatie op basis van morfologische kenmerken.
De doelstellingen van deze studie zijn het identificeren van de Peruaanse stalen, het detecteren van een fylogenetisch signaal in de alkaloïde productie en het ontdekken van fylogenetische patronen in *Cinchona* spp. in een uitgestrekt geografisch gebied in hun regio van herkomst, Peru, waar we een hoge genetische variabiliteit in de populaties verwachtten. Door de genetische diversiteit van elke bemonsterde populatie te karakteriseren, hebben we prioritaire *Cinchona* populaties kunnen aanduiden voor conservatie. Het aantal stalen in deze studie was echter beperkt en onze gegevens waren niet ideaal voor populatie-genetische studies. Daarnaast wilden we de identiteit van gecultiveerde accessies afkomstig uit plantages in Zuid-Kivu, DR Congo achterhalen, hun oorsprongsgebied bepalen en de genetische diversiteit tussen plantages bepalen. Genetische profielen van *Cinchona* accessies werden gegenereerd met behulp van drie chloroplast merkers en één nucleaire merker.
We identificeerden de populaties van Jaén en Bongara in Peru op soortniveau als *Cinchona pubescens*, terwijl de Peruaanse populaties uit San Ramón en Rodríguez de Mendoza wel tot het geslacht *Cinchona* behoren maar niet werden geïdentificeerd als *C. pubescens*. Populaties in Bongara en Rodríguez de Mendoza vereisen de hoogste prioriteit voor conservatie omdat deze populaties de hoogste genetische diversiteit bevatten. Desalniettemin dient elke bestudeerde populatie beschermd te worden tegen verdere ontbossing en vernietiging door uitbreiding van de landbouw. De gecultiveerde accessies uit DR Congo werden geïdentificeerd als *C. calisaya*. Ons resultaat suggereert dat deze accessies een hoge similariteit vertonen met inheemse populaties uit Bolivia. Om de oorsprong van de gecultiveerde *C. calisya* accessies specifieker te bepalen, is een bredere bemonstering van het volledige inheemse verspreidingsgebied in Peru en Bolivia noodzakelijk. Bovendien is het gecultiveerde genotype gelijk aan het laagproductieve *C. calisaya* genotype in Bolivia. Desalniettemin blijven de invloed van het genotype en de omgeving op de kinine concentratie in *Cinchona* echter onbekend. Deze studie kan funderen als basis voor het bepalen van fylogenetische patronen in de productie van alkaloïden in *Cinchona*.

Dedication

To mum and dad, *Vera* and *Kris*, I would like to thank you for all the support you gave me during my Bioscience Engineering studies at Ghent University. Without your encouragements, I would never have completed an exchange visit to Australia and a field work campaign in Peru during my master's degree in Cell and Gene Technology at Ghent University. I am very grateful for your unconditional support and love. I am also extremely thankful that I could write my thesis and finish my courses at home in Zottegem with you during the COVID-19 lockdown. It was very nostalgic for me spending so much time at home and very unique having you around all the time, which I really enjoyed. I also want to thank my grandmother, also being my godmother, *oma Mariette*. Being part of my bubble during the COVID-19 lockdown, she always cheered for me while I was doing my workouts at her place across the street. I also want to thank my little spoiled brat, but adorable sister, *Alina*.

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Contents

Abbreviations

CO1 cytochrome *c* oxidase subunit 1-gene

a.s.l. above sea level

cpDNA chloroplast DNA

DBH Diameter at Breast Height

GBS Genotyping-by-Sequencing

HTS High-Throughput Sequencing

mtDNA mitochondrial DNA

nrDNA nuclear ribosomal DNA

PCR polymerase chain reaction

rDNA ribosomal DNA

rRNA ribosomal RNA

RRS Reduced representation sequencing

SLA specific leaf area

SNP single nucleotide polymorphism

WGS whole genome sequencing

Part I

Context

1 Problem statement

Malaria, caused by the parasite *Plasmodium* sp., remains one of the most prevalent diseases in the world. In 2018, an estimated 228 million cases of malaria were registered, of which 93% occured on the African continent (WHO, 2019). Malaria can be treated with chemical substances that inhibit the growth of *Plasmodium*. Quinine is the first effective and thus the oldest anti-malarial cure available. It is obtained from the bark of the *Cinchona* tree. Nowadays, synthetic compounds such as chloroquine are also used to treat malaria. Although these drugs were initially more effective against malaria than quinine, the evolution of resistance in *Plasmodium* against these compounds strongly reduced their usage. In addition, quinine is a relatively inexpensive drug and quite often the only available treatment in African countries.

Cinchona trees naturally occur on the border between the Andean mountains and the rainforest along the western edge of South America. Deforestation and overexploitation of *Cinchona* trees have caused that wild *Cinchona* populations became at risk of extinction. Data on the current distribution of *Cinchona* in South America is limited. Most studies focused on Colombian and Ecuadorian samples (Hodge, 1948; Andersson, 1998). Taxonomic classification of *Cinchona* species remained an endless debate throughout history due to the difficult identification of species based on morphological characteristics.
The species *C. pubescens* and *C. calisaya* have been cultivated in several countries in South America, Asia, and Africa. Smallholders in DR Congo are responsible for 25 % of the world quinine production. However, quinine production in DR Congo is assumed to be suboptimal due to the use of genotypes that produce lower yields under the given environmental conditions. However, the influence of the genotype and the environment on the quinine concentration in *Cinchona* trees remains unknown.
Research on genetic and environmental factors that determine quinine concentrations in *Cinchona* is limited so far due to the emergence of synthetic alternatives and the decolonisation. An executive examination of the relative effect of the genotype and the environmental diversity in determining the plant secondary metabolite production under natural conditions was performed on *C. calisaya* in Bolivia (Maldonado et al., 2017). Nevertheless, studies that analyze both effects in other *Cinchona* species in its native range in Central and Northern Peru are lacking.

2 Objectives of this study

This study aims to use a phylogenetic approach to investigate the variation in quinine production within and between *Cinchona* species in their native and introduced range.
This study focuses on the geographic distribution of *C. pubescens* in Central and Northern Peru. We want to reveal phylogenetic patterns in *C. pubescens* along a broad geographic range in its natural habitat, where we expect high genetic variability. When genetic variability can be assessed, we want to detect a phylogenetic signal in the alkaloid production. In addition, we aim to identify the region of origin of the cultivated species in DR Congo and estimate the genetic differentiation between those cultivated accessions and accessions from its native range.
This project contributes to a sustainable intensification of the *Cinchona* cultivation and the conservation of *Cinchona* populations in the tropics.

3 Impact COVID-19 on Master Dissertation

The COVID-19 crisis impeded the laboratory work that had to be performed on the *Cinchona* bark samples. The optimisation of the extraction protocol of the four major alkaloids could not be performed and the extraction of these alkaloids was not yet carried out for this reason. The results of the laboratory analysis were supposed to be included in this master dissertation, which was not possible due to the closure of the laboratory. This has an impact on the results of this study, as the potential phylogenetic signals in the alkaloid production could not be assessed. This dissertation would have primarily focused on creating a phylogenetic tree and analysing the effect of phylogenetic variation on the alkaloid concentration. The measurements that were taken as part of the COVID-19 crisis shifted the emphasis towards a population genetic study of *Cinchona* in its native and introduced range. However, our sampling was not entirely appropriate for an elaborate population genetic study on *Cinchona*, as samples were collected in function of our original research objectives. This preamble was drafted by the student and the supervisors and approved by both.

Part II

Literature study

Chapter 1

Introduction

The national tree of Peru belongs to the genus *Cinchona*, the second-most economically valuable plant genus in the coffee family (Rubiaceae) after the coffee genus (*Coffea*). The national tree is visualized in the upper right section of the Peruvian national emblem, as seen on every Peruvian coin and the Peruvian flag, shown in Figure 1.1. The *Cinchona* genus comprises about 23 different species of trees and shrubs, which are native to the tropical Andean forests of western South America and Central America at an altitude between 800 and 3300 *m* above sea level (a.s.l.) (Jäger, 2004). Figure 1.2 visualizes the distribution of *C. pubescens* and *C. calisaya* in their native range.

The Swedish botanist Linnaeus named the genus *Cinchona* after the wife of the viceroy of Peru, the **Countess of Chinchón** in 1742. The Countess fell ill with a tertian fever and was cured by a treatment with the red bark of *Cinchona*, while all other treatments failed to help her. Even though this story appeared to be untrue, *Cinchona* bark was frequently administered in the early seventeenth century for intermittent fevers coinciding with a malaria infection that is caused by five different *Plasmodium* species. Although malaria was not present in Latin-America before the European colonization, native Americans also used the bark of *Cinchona* to relieve fevers (Jaramillo-Arango, 1950). In Europe, Jesuits were particularly interested in the bark, as the area around Rome was known for its malarial swamps. The Jesuits distributed the red bark, also known as the **Jesuit's bark**, to Europe in 1631 (Rocco, 2003).

The bark and the roots of *Cinchona* species contain important alkaloids, such as **quinine**, which were used to treat malaria between the 17th and 20th century. Due to deforestation and overexploitation of *Cinchona* for its medicinal uses, multiple species are in danger of extinction in its native range. Numerous *C. pubescens* and *C. calisaya* plantations were established in tropical regions all over the world (e.g. Indonesia, India, Sri Lanka, DR Congo, Mexico) by smuggling seeds from its native distribution area (Andersson, 1998).

Figure 1.1: The current state flag of Peru, which was officially adopted on February 25, 1825. The Peruvian emblem visualizes a vicuña (Peru's national animal), a *Cinchona* tree (native to tropical Andean forests and source of the anti-malaria drug quinine) and the horn of plenty.

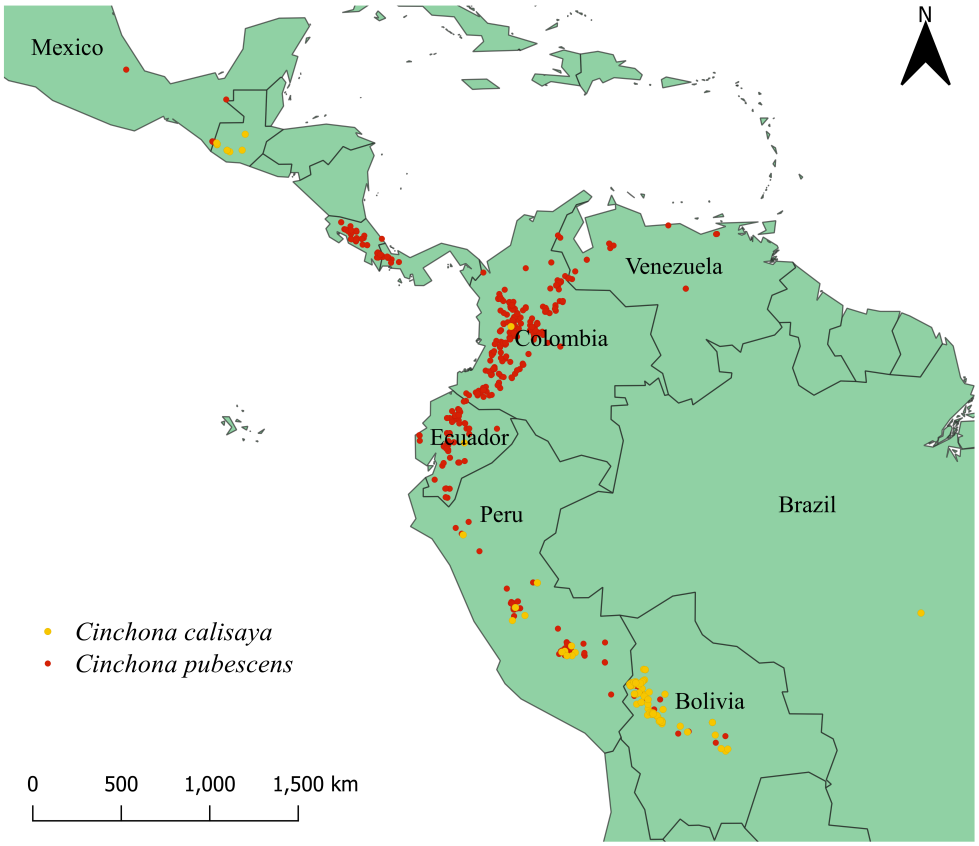


Figure 1.2: Natural distribution area of *Cinchona pubescens* (red dots) and *Cinchona calisaya* (yellow dots) in Central and South America. The indicated *Cinchona calisaya* trees in Brazil, Guatemala, and Colombia are presumably non-native (GBIF, 2019).

Chapter 2

Cinchona: the study species

2.1 Taxonomy

In 1738, **La Condamine** was the first who described the genus *Cinchona*, which was frequently known to Europeans by the name *Quina-Quina* (de La Condamine, 1738). The name *Quina-Quina* dates from the second work of **Sebastiano Bado** (Bado, 1663). However, this name was mistakingly linked to *Cinchona* trees by Sebastiano Bado, as Quina-Quina was originally used by the South American Quechua people to refer to *Myroloxon peruiferum*, the Peruvian balsem tree. *Quina-Quina* had a totally different etymology and was used erroneously throughout history. This had a widespread effect upon the historical literature of the subject, as described in the work of Haggis, which denounces as well the romantic stories about the discovery of *Cinchona* and its introduction into Europe (Haggis, 1941). **Linnaeus** named the genus *Cinchona* L., after the Countess of Chinchón in 1742 (Linnaeus, 1742).

The hybridization of different *Cinchona* species in nature makes taxonomy of the genus extremely difficult (Jäger, 2004). Because of its medicinal properties, taxonomists were very interested in the genus *Cinchona* and new names were created based on small morphological differences. In 1998, **Andersson** revised more than 330 names and recognized only 23 valid species (Andersson, 1998). This deduction was based on a cladistic method using 48 morphological characters (Andersson, 1995).

One of the 587 equally parsimonious trees from Andersson is visualized in Figure 2.1. This phylogenetic tree contains 22 out of 23 known Cinchona species and a single species of the genera *Ladenbergia*, *Remijia*, and *Joosia*, serving as outgroup in the tree. The genus *Cinchona* and the genera in the outgroup belong to the Rubiaceae family in the major group of angiosperms (flowering plants). Within this family, these genera are classified into the subfamily Cinchonoideae and the tribe Cinchoneae. The latter includes seven described genera (*Ciliosemina*, *Cinchona*, *Cinchonopsis*, *Joosia*, *Ladenbergia*, *Remijia*, and *Stilpnophyllum*) that all occur in the Neotropics,

predominantly in the Andes (Bremer & Eriksson, 2009). The economically most important genus within the Rubiaceae is *Coffea*, used for the production of coffee.

Most *Cinchona* species occur in restricted areas and are thus ecologically differentiated (mainly in relation to altitude) in their native range. For this reason, the natural range of most species do not overlap and only a few species grow in close distance of one another (Andersson, 1998). As shown in Figure 1.2, *Cinchona pubescens* is widely distributed in Central and South America. Spontaneous hybrids are especially known between *C. pubescens* and other more restricted species. Andersson mentions seven species that hybridize with *C. pubescens*: *C. micrantha*, *C. calisaya*, *C. barbacoensis*, *C. macrocalyx*, *C. lucumifolia*, *C. officinialis*, and *C. lancifolia*. Populations of hybrids of *C. pubescens* and *C. calisaya* have been cultivated and are most commonly found in nature (Andersson, 1998).

Numerous synonyms are listed for *C. pubescens* by various sources. The four major ones are *C. chomeliana*, *C. lutea*, *C. ovata*, and *C. succirubra* (Jäger, 2004). *Cinchona ledgeriana* is a synonymous name for *C. calisaya* (GBIF, 2019).

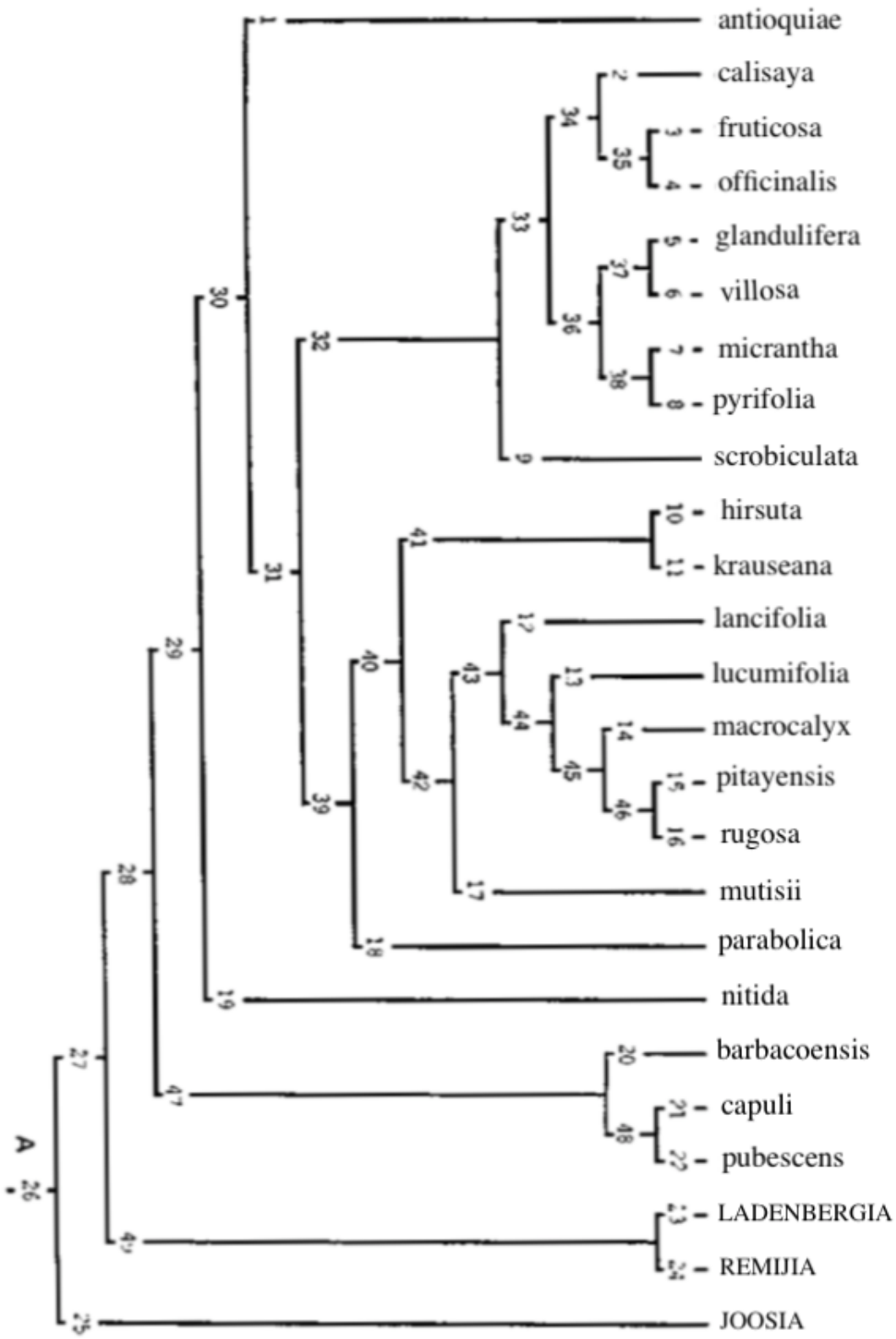


Figure 2.1: Phylogeny of *Cinchona*, one out of 587 equally parsimonious trees from the cladistic procedures. *Ladenbergia*, *Joosia* and *Remijia* are represented by only one species each: *L. oblongifolia, J. dielsiana*, and *R. pedunculata* (Andersson, 1998).

2.2 Morphology

2.2.1 Leaves and leaf color

The leaves of all *Cinchona* species are opposite, stipulate, petiolate, and entire-margined, as can be seen in Figure 2.2. There is a lot of variation in texture and surface structure, which are useful characters for taxonomy within the genus (Andersson, 1998). Leaves of saplings and other rapidly growing shoots are usually much larger than from flowering shoots. The taxonomy of Andersson is mainly based on leaves of flowering shoots. The texture of leaf blades varies from membranous to coriaceous. *C. pubescens* is characterized by membraneous, large and broad leaf blades and a 2-layered epidermis with a thin cuticle. The leaves are elliptic to nearly orbicular with petioles (1.2 - 5 cm). The leaves usually have a width between 5.3 cm and 21 cm and a length between 8.3 cm and 23 cm. There are mostly caducous stipules (1.2-2.6 x 0.5-1.5 cm), which leave a scar on the branch (Acosta Solís, 1945). The glossiness of the leaf surface, which can be either matte or glossy in *Cinchona*, cannot be used to distinguish species, as it is inconsistent with the states of other characters probably due to hybridization. The leaf blades of *C. pubescens* consist out of 7-11 pairs of conspicuous secondary veins. The leaf domatia are pouch-shaped, but usually absent (Andersson, 1998).
Another taxonomic character is the nature of the indumentum of the abaxial leaf blade surface, which is the hairy covering of plants, as many species (*C. calisaya* and *C. pubescens*) vary a lot in the amount of indumentum. Most species do not have hairs on the abaxial leaf surface. However, introgression with *C. pubescens* can lead to a hairy morphotype (Andersson, 1998). The hairy abaxial leaf surface of *C. pubescens* is mostly visible on young leaves.
Small variations in size and shape seem to be not important on taxonomic level. Reasons for this variation might be local differentiation and clinal variation in response to altitude (von Humboldt, 1808). This variation is even increased because of hybridization in nature, mainly with *C. calisaya* and *C. macrocalyx* (Andersson, 1998). Leaves turn red when they become older to protect themselves of sun damage (Figure 2.2b) (Cedeño, 1990; Rentería, 2002).

2.2.2 Flowers, fruits and seeds

Merosity in flowers refers to the number of sepals, petals, stamens, and/or carpels in a distinct whorl structure of a plant. The **flowers** of *Cinchona* species are usually pentamerous, which refers to a whorl of five parts. They are spreaded in a loose, irregular branching cluster up to 20 cm long or slightly longer. Flowers with a different merosity only occur in low numbers among other pentamerous flowers (Andersson, 1998). As noted by Andersson, the largest pyramidal inflorescence was registered for *C. pubescens*, where a size of 50 x 50 cm is reached. Some of

(a)

(b)

Figure 2.2: (a) Young seedlings of *C. pubescens* in Galápagos (Jäger, 2004). (b) Leaves of *C. pubescens* in Galápagos (Jäger, 2004).

the species, such as *C. pubescens*, *C. nitida* and *C. antioquiae* were registered to be strongly fragrant. It is not known if the lack of scent of other species of the *Cinchona* genus is real or due to the collector's failure to note. Flowers have a small, tubular at the base, and distinctly lobed calyx. The size of the calyx can be used as a taxonomic character and is ca. 1 *mm* in *C. pubescens*. The calyx is usually smooth inside and sometimes soft down at the base, which is a variable feature among species. The corollas are usually thick-structured and have short appressed hairs on the outside, apart from the calyx. Because of the pubescence at the outside, they have a greyish pulverised appearance. The color of the corolla might vary between species and can have various shades of red as well as white. White flowers are common for *C. calisaya* and *C. micrantha*. The flowers of *C. pubescens* vary from pink to purple (Figure 2.3) (Andersson, 1998). The lobes of *Cinchona* species are not keeled, as is the case for *Ladenbergia* and *Remijia* species, but rather flat ventrally (Andersson, 1995). A marginal indumentum is found on the inside of the corolla lobes, which is characteristic for the genus (Andersson, 1998). The stamens in the bisexual flowers are inserted in the corolla tube. The flowers are heterostylous, as there are multiple morphological types of flowers, termed "morphs". The styles are always smooth in *Cinchona* species, which is not the case for *Ladenbergia* and *Remijia* species (Andersson, 1998). Flowers of *C. pubescens* develop all year round, with an extremum from august until november (Rentería, 2002).

The **fruits** are elliptical to subcylindrical or subglobular capsules. In *C. pubescens* and *C. antioquiae*, they are typically flattened cylindrical and 13-41 x 5-7 *mm* long (Figure 2.3) (Andersson, 1998). During maturation, the capsules open from base to tip and the fruit splits into two valves. Each capsule comprises about 60-70 seeds. It takes about 19 weeks between the

Figure 2.3: Morphological characteristics of *Cinchona pubescens* in Galápagos. Top left: Flower panicles, top center: detail of flower, top right: fruits with Ecuadorian 10-cent coin (ca. 1.8 cm), bottom left: opened flowers, and bottom right: seeds (scale in cm) (Jäger, 2004).

(a)

(b)

Figure 2.4: (a) Drawing of the morphologic characteristics of *C. pubescens* (Köhler, 1897). (b) Drawing of a seed of *C. pubescens* with the basiscopic end down (Andersson, 1998).

opening of the flower and the production of mature fruits (Rentería, 2002). A useful taxonomic character is the thickness of the endocarp. *C. pubescens* and *C. calisaya* usually have a thin-walled endocarp (0.1 - 0.2 mm) (Andersson, 1998).
Seeds of *Cinchona* species are winged, planoconvex and 7-12 x 2.1-2.8 mm, which includes the irregular dentate wings. These irregular wings are a useful taxonomic character (Andersson, 1998). An illustration of the seed of *C. pubescens* is given in Figure 2.4b.

2.2.3 Wood, bark and its alkaloids

Knowledge about wood characteristics in *Cinchona* was rather limited, because the economic interests in this trait were low. The color of the wood of *C. pubescens* varies between brown/yellow and brown/orange. The wood is compact and has a density of 0.54 g/cm^3 (Chave et al., 2009). The thickness of the coffee-brown outerbark is between 3-20 mm. There can be white streaks on the bark, because of lichens which grow on the outer surface of the tree (Acosta Solís, 1945). A milky fluid appears when some of the outer bark is taken off and the cortex below turns red, a reference to the given name "red bark" of the species *C. pubescens* (Nair, 2010). Whereas *C. calisaya* is known as the "yellow bark" (Maldonado et al., 2017). The bark contains pigments (up to 10 %), polyphenols, flavonoids, essential oil, and alkaloids (Nair, 2010). An important characteristic for all *Cinchona* species is the bitter taste of the bark because of the presence of a class of chemical compounds called alkaloids (Acosta Solís, 1945).

Alkaloids

Alkaloids are a diverse group of low-molecular weight and nitrogen-containing molecules. They are found in about 20 % of the plant species and play many roles in nature (Zulak, Liscombe, Ashihara, & Facchini, 2006). These secondary metabolites are not directly involved in the primary metabolism (i.e. growth and cell function maintenance) and are thought to play an important role in the defense against herbivores and pathogens. Alkaloids have a wide range of biological activity including antimalarial (e.g. quinine extracted from *Cinchona*), antiasthma (e.g. ephedrine extracted from *Ephedra*), anticancer (e.g. homoharringtonine extracted from *Cephalotaxus*), antiarrhythmic (e.g. quinidine extracted from *Cinchona*), etc. Many of them are extracted and exploited because of their pharmacological activities (Wink, 2015).

The *Cinchona* bark comprises up to 16 % by mass of quinoline alkaloids (Tsimachidis, Česla, Hájek, Theodoridis, & Jandera, 2008). All these alkaloids contain quinoline and quinuclidine rings with a vinyl group attached to it, as can be seen in Figure 2.5. The major quinoline alkaloids of *Cinchona* are quinine, quinidine, cinchonine, and cinchonidine, which are visualized in Figure 2.5. Apart from these, 30 other compounds associated to quinine are present in the *Cinchona*

bark in minor quantities. Quinine is a dextrorotary isomere of quinidine. Both molecules contain a methoxygroup which fluoresce in ultraviolet light. Cinchonine and cinchonidine do not contain this group and, therefore, do not fluoresce in ultraviolet light (Nair, 2010).

The concentration of these alkaloids is low in twigs, higher in the bark, and highest in the roots. The highest concentration in the bark is found around 30 - 45 cm from the base of the tree. The relative concentrations of these alkaloids vary between different species and usually increase with age (1-12 years) (GIBBS, 1885; Chopra & Peter, 2005). The exact role of *Cinchona* alkaloids is unknown. Studies have shown that the production of the alkaloids is upregulated in the presence of insects in order to inhibit their potential harmful activity on the tree (Green, Simmonds, & Blaney, 2002). *Cinchona* alkaloids are usually stored in plants as salts of quinine and as cinchotanic acids. It was shown in plantations in Bolivia and India that the highest alkaloid concentrations were established in the bark of the plants between the fourth and seventh year and then declined again (GIBBS, 1885; Chopra & Peter, 2005). The alkaloid concentration varies between seedlings from the same variety due to soil and environmental conditions. *C. ledgeriana* (syn. *C. calisaya*) for instance had a higher bark alkaloid concentration if this species was grown at high soil nitrogen concentrations (Loustalot & Winters, 1948). The quinine content in the bark can be increased by impeding vertical growth (Coster, 1942). Quinine is the major alkaloid in the cultivated species *C. calisaya* and *C. officinalis* (Table 2.1), while cinchonine is the major alkaloid in *C. pubesecens* comprising 3.3-4 % of the dry weight. The total alkaloid concentration in *C. pubescens* varies between 6.0-8.2 % of the dry weight (Nair, 2010).

Table 2.1: A comparison of quinine content in various *Cinchona* species (Hunter, 1988; Martin & Gandara, 1945).

Species	Typical range of quinine content in dried bark (%w/w)
C. calisaya	3-4
C. officinalis	1.5-3
C. pubescens	0.8-2

Although *C. calisaya* has the highest quinine content, *C. pubescens* is more often cultivated, because of its wider range of growth conditions, high robustness against biotic and abiotic stress and its ease to propagate (King, 1880). *C. pubescens* is often used in Indonesia as a root stock for the grafting of *C. calisaya*, because it is hardier and can grow better at lower elevations (Chopra & Peter, 2005). High-performance thin-layer chromatography (HPTLC) determines simultaneously, rapidly, and accurately the four alkaloids in the bark of *Cinchona* species (Dayrit, Guldotea, Generalao, & Serna, 1994).

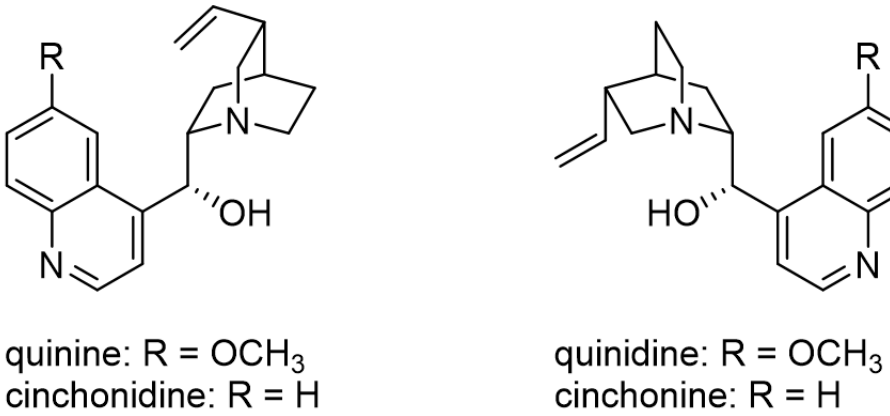


Figure 2.5: Chemical structure of the four major alkaloids in the bark of *Cinchona* trees: Quinine, Cinchonidine, Cinchonine, and Quinidine.

2.3 Natural distribution

Cinchona species naturally occur on the slopes of the Andean mountains at an altitude between 800 and 3000 *m* a.s.l. along the western side of South America (Nair, 2010). The species *C. pubescens* is widely distributed from the highlands in central Costa Rica and Panama to the maritime region in North-East Venezuela, and in the Andes regions of Colombia, Ecuador, Peru, and Bolivia (Figure 1.2) (Andersson, 1998).
Cinchona trees became rare in their natural environment due to overexploitation of the trees for the production of *Cinchona* alkaloids and due to deforestation (Acosta Solís, 1945). In 1859, *C. pubescens* was almost completely vanished in Ecuador and other regions (Spruce, 1861; N. Taylor, 1943).

2.4 Ecology

Some *Cinchona* species, which usually occur in dry lowlands or at altitudes between 300 and 3300 *m*, grow as shrubs (e.g. *C. fruticosa* and *C. krauseana*). However, most *Cinchona* species grow as trees with an average height of 18 *m* and an average diameter at breast height (DBH) of 18 *cm* (Andersson, 1998). *C. officinalis* has been described as a shrub or a small tree. In the north of Peru, *C. pubescens* occurs as a dominant tree species with a height of 30 *m* and a DBH of 90 *cm* (Hodge, 1948). *C. calisaya* has been described as a liana, a slender tree, or a shrub. It can be noted that the growth form is related to the habitat where the species is located (Andersson, 1998).
In their natural environment, *Cinchona* trees optimally grow in humid and warm conditions with a high precipitation intensity almost all year round and a mean annual temperature be-

tween 10 and 23°C (Acosta Solís, 1945; Garmendia, 2005). Trees grow in clay soils and rocky areas. The composition of the soil is usually high in organic material, volcanic ash, nitrogen and phosphorous, but low in potassium. The temperature of the soil is on average 16.3 °C and thus warm, the pH is slightly acidic (on average 5.1) (Acosta Solís, 1945; Garmendia, 2005). Studies in cultivated areas in India, Guatemala, and Indonesia confirmed that *Cinchona* trees grow best in soils which are well precipitated and contain a high amount of organic material. In addition, *Cinchona* trees prefer a high nitrogen content, a low carbon/nitrogen ratio, and high levels of soluble citric phosphate, lime, and hydrolytic acids (Chopra & Peter, 2005; King, 1880; Coster, 1942; Popenoe, 1949). *C. pubescens* usually occurs in disturbed areas and at steep gorges as protection against erosion (Acosta Solís, 1945). The trees also grow better in a recently cleared forest in comparison to grassland (King, 1880). It has been reported that *C. pubescens* trees of plantations in Bolivia grow best at an altitude of 900-1200 *m*. At higher altitudes, the growth form is stunted and quinine concentrations are lower (GIBBS, 1885). Optimal altitudes in plantations in India are between 460 and 2000 *m*, in Tahiti between 600 and 950 *m* in primary and secondary forests, in São Tome en Principe between 650 and 1350 *m*, and in Java between 1250 and 1950 *m* (Chopra & Peter, 2005; Vanquin, 2006; Engler, 1896).

Although the general plant architectural details are unknown for the genus, Andersson observed that *C. pubescens* grows monopodial, which is a character that gets lost during early growth stages (Andersson, 1998).

Wild *Cinchona* species are mainly associated with other tree species, such as *Palicourea* spp., *Joosia pulcherrima*, *Hoffmania ecuatoriana*, *Hieronyma macrocarpa*, *Ocotea* spp. and *Weinmannia fagarioides* (Acosta Solís, 1945). In Peru, *C. pubescens* co-occurs at higher altitudes (ca. 2445 *m*) with *Miconia* spp., *Clusia* spp., *Weinmannia pinnata*, *Hedyosmum scabrum*, *Chusquea scandens*, *Ilex* spp. and *Hesperomeles* spp. At lower altitudes, *C. pubescens* is predominantly associated with trees of other genera, such as *Clusia*, *Befaria*, *Wemmania*, and *Brunellia* (Alonso, Alonso, Schulenberg, & Dallmeier, 2001).

2.5 Commercialization of *Cinchona* species

The natural populations in the Andes were continuously exploited and tons of bark were exported to different parts of the world before the advent of the chemical synthesis of quinine in 1944 (Nair, 2010; Woodward & Doering, 1945). Figure 2.6 demonstrates the spread of *Cinchona* species in the Andes for commercial purposes. Although *C. pubescens* occurs throughout the whole range of the genus, it was only exploited in Colombia, Ecuador, and Peru (Hodge, 1948).

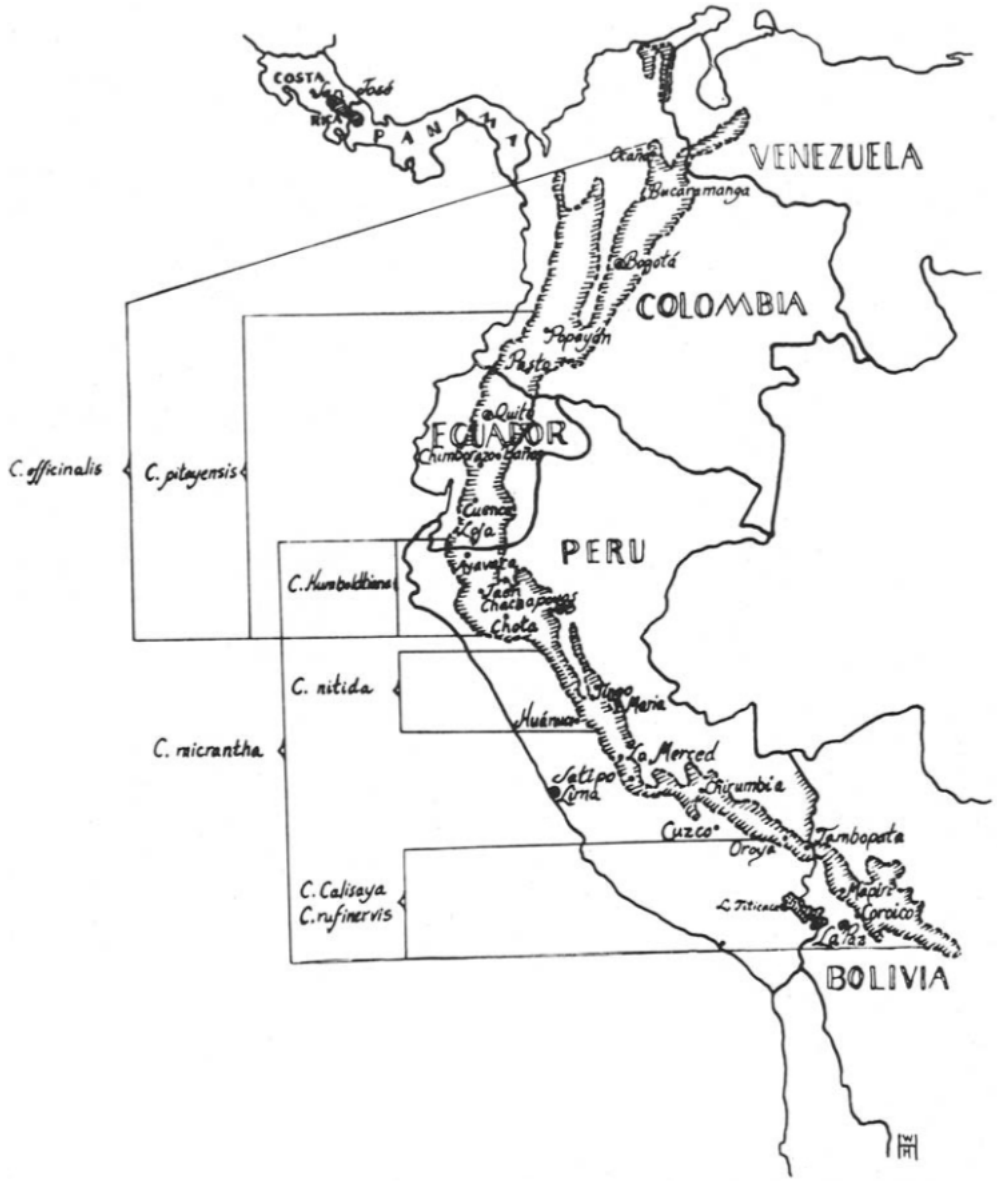


Figure 2.6: Map demonstrating the distribution of the *Cinchona* species used for commercial purposes in the Andes. *C. pubescens* grows throughout the whole range of the genus, but was only exploited in Colombia, Ecuador, and Peru. The brackets at the left indicate the individual range of each species and the cross-hatched area indicates the overall-range of the genus (excluding *C. amazonica*). The major centers for *Cinchona* bark are cross-hatched (Hodge, 1948).

As the demand for the bark rapidly increased in Europe and the United States, European naturalists (e.g. Alexander von Humboldt) featured that the natural stocks soon would run out and warned for the harmful harvesting methods of the *Cinchona* bark. They appealed for the search of *Cinchona* saplings and seeds to export them to their colonies for cultivation (Crawford, 2009; Rocco, 2003; Headrick, 1988; Roersch van der Hoogte, 2015). In 1852, the first *Cinchona* bark tree was introduced in the mountain valley of Tjibodas in Java, an island of Indonesia (Headrick, 1988). According to Headrick, this plant originated from *C. calisaya* seeds that were smuggled out of Bolivia and grown in the botanical garden in Leiden (Headrick, 1988). Several trial and error projects, performed by Dutch-state funded pharmacists and chemists, resulted in the introduction, acclimatization, and commercialization of *Cinchona* species in Java. Multiple plantations were founded by the Dutch, British, and French empires in tropical regions all over the world (e.g. Indonesia, Sri Lanka, India, DR Congo, Mexico) and the search for the most productive *Cinchona* trees started (Andersson, 1998). By the end of the nineteenth century, the Dutch colonies in the Netherlands Indies were important leaders on the international market for *Cinchona* bark production and export, as their export exceeded the one from countries exploiting their natural resources (Roersch van der Hoogte, 2015).
The most productive known *Cinchona* species is *C. calisaya*, also known as the yellow bark tree from Bolivia (Nair, 2010). Although the bark of *C. calisaya* contains the highest quinine concentration (Table 2.1), *C. pubescens* has the largest amount of bark, especially the accessions grown in Java and Sri Lanka. Therefore, *C. pubescens* was chosen as the species for cultivation in 1891 (Schumann, 1891). Moreover, *C. pubescens* is tougher, easier to breed and has a broader spectrum of growth conditions (King, 1880). Nowadays Indonesia, the Democratic Republic of the Congo, Tanzania, Rwanda, Kenya, Sri Lanka, Bolivia, Colombia, Costa Rica, and India are the countries with the highest production of *Cinchona* alkaloids. Together, these countries produce 400-700 tons of alkaloids from 8,000-10,000 tons of bark per year (Husain, 1991; Franckenpohl, 2000). In India, an annual yield of 9,000-16,000 kg bark per hectare is obtained (Bose, Kabir, Das, & Joy, 2001).
In addition, *Cinchona* species were grown in several Pacific islands, such as the Galápagos, Hawaii, and Tahiti. In these countries, *Cinchona* occurs as invasive species, as they never have been exploited for their medicinal features (Meyer, 2004; Jäger, Kowarik, & Tye, 2009; Weber, 2017).

2.5.1 *Cinchona* cultivation in DR Congo

During the first four decades of the twentieth century, the plantations in the Dutch East Indies were critical for the worldwide supply of *Cinchona* alkaloids. However, World War II was a turning point for the Dutch quinine production. The globalization of the *Cinchona* production

induced the rise of *Cinchona* plantations in Belgian Congo (Congo Belge) in 1933, which are still important on the international market today (van der Hoogte & Pieters, 2016). In 1899, experiments for *Cinchona* cultivation were done for the next few decades by the Belgian colonial government in the Kivu region (B.C.B., 1954). According to Rocco (2003), seeds of Congolese plantations originated from a donation of the Dutch Queen Juliana to the Belgium King Albert I, father of Prince Leopold III. A tin of *Cinchona ledgeriana* (currently classified as *C. calisaya*) seeds was smuggled from Bolivia to Java by Charles Ledger and Manuel Incra Mamani and seeds were dispatched to Queen Juliana by Dutch planters from Java. The donated seeds were distributed to plantations in the region of Bukavu (Rocco, 2003). The adequate environmental conditions, a sandy loam soil and moderate precipitation, allows the species to thrive along the mountainous slopes in the region, that resemble the conditions of the species' natural habitat in the Andean mountains (Roersch van der Hoogte, 2015).
The most extensive association of *Cinchona* producers in DR Congo, named the *Société Coopérative "Congokina"*, invested in the expansion of the *Cinchona* production and established their own quinine factory in Bukavu, Pharmakina (Roersch van der Hoogte, 2015). According to the representative of Pharmakina, Desso Kaningini, the plantations of the company cover 3800 ha in Kivu and 200 ha in Rwanda (Malu-Malu, 2013). The *Cinchona* production remains suboptimal nowadays in DR Congo and the search for more reproductive species to increase the quinine yields is still ongoing (Kibriya, Partida, King, & Price, 2014).

2.5.2 *Cinchona* cultivation and its threats

Only seven out of 27 fungi species that are listed in the database of the Systematic Mycology and Microbiology Laboratory (e.g. *Elsinoe cinchonae* Jenkins, *Phytophthora cinnamomi* Rands, *Prillieuxina cinchonae* J.A. Stev.) appear in *C. pubescens*' native range, but none of these species have been recorded as an economic threat (Jäger, 2004; Farr & Rossman, 2020; PIER, 2009).
However, several pests and diseases are threatening *Cinchona* production in cultivated areas (e.g. in India, Guatemala, and Java). The disease "damping off" is caused by the fungi *Rhizoctonia solani* J.G. Kühn, *Phytophtora* spp. (root rot fungus), and *Rosellinia arcuata* Petch (black-root fungus) that affect the *Cinchona* seed beds (Chopra & Peter, 2005; Coster, 1942; Popenoe, 1949). *Cinchona* species are most vulnerable during their early growth stages, and an optimal control of these stages is of utmost importance. Insects, such as *Helopeltis* sp. (tea mosquito) and *Pachypeltis* sp. (leaf scorch), have also infested plantations in Java and India (Chopra & Peter, 2005; Coster, 1942). An important disease in plantations in Rwanda and DR Congo is the *Cinchona* stem bark disease caused by *Phytophthora cinnamomi* Rands (Chopra & Peter, 2005). *Fusarium* spp. and *Botryodiplodia theobromae* Pat. are registered as secondary pathogens in its introduced range (e.g. in Galápagos) (Jäger, 2004).

Chapter 3

Cinchona and its applications

3.1 The medicinal use of *Cinchona* alkaloids

The bark of *Cinchona* was used to extract alkaloids, such as **quinine**, as the first effective cure to treat malaria. Malaria is the most common disease in Africa and some countries in Asia (WHO, 2019). It is caused by a parasite belonging to the *Plasmodium* genus (Escalante & Ayala, 1994). The disease is transmitted to humans by an infected female *Anopheles* mosquito. Five out of 172 *Plasmodium* species infect humans and cause the disease malaria (*P. vivax*, *P. malariae*, *P. falciparum*, *P. ovale*, and *P. knowlesi*) (Antinori, Galimberti, Milazzo, & Corbellino, 2012). *Plasmodium* species are intracellular parasites that accumulate an insoluble metabolite of heme digestion, often called malaria pigment. They live in several vertebrates, some in tissues, some in red blood cells (Talapko, Škrlec, Alebić, Jukić, & Včev, 2019). The pathogenesis of malaria is associated with fever and chills due to the rupture of erythrocytic-stage schyzonts. In severe *P. falciparum* infections, infected red blood cells may obstruct capillaries, leading to hypoxia and the release of toxic cellular molecules (Crutcher & Hoffman, 1996). Pregnant women, children, immunocompromised individuals, and healthy people who have never encountered *Plasmodium* infection are extremely vulnerable to malaria (Talapko et al., 2019). For this reason, prophylaxis is recommended in malaria endemic areas (WHO, 2019).

Quinine possesses rapid activity against the blood stages of intra-erythrocytic malaria parasites (Warhurst, Craig, Adagu, Meyer, & Lee, 2003). The four major alkaloids, visualized in Figure 2.5, all posses antimalarial activity, but they have a different pharmacological profile and different pharmacological properties (Berliner et al., 1948; Hill, 1963; Bruce-Chwatt, 1990). Out of the four major alkaloids found in the *Cinchona* bark, quinine is the most potent one to cure malaria (Nair, 2010). Quinine interferes with the development of the mature trophozoite stage of *Plasmodium* parasites (Rieckmann et al., 1968). It interferes with the parasite's heme metabolism and blocks the detoxification of heme molecules. The quinolone rings of quinine intercalate between the

aromatic groups of ferriprotoporphyrine molecules, hampering the formation of hemozoin crystals, also called malaria pigment (Weissbuch & Leiserowitz, 2008). Quinine is highly active against several protozoa, it is also bacteriostatic and it inhibits yeast fermentation (Nair, 2010). The efficacy of quinine against malaria resulted in the confusing assumption that this active substance has antipyretic (reducing fever) properties, which has been disproved by several studies (Krishna et al., 1995). However, quinine does possess analgesic (relieving pain) properties (Achan et al., 2011).

Before 1820, dried bark was grounded and mixed into a beverage (Izawa, Amino, Kohmura, Ueda, & Kuroda, 2010). In 1820, quinine was isolated from the other alkaloids as a quinine sulfate by Pierre-Joseph Pelletier and Joseph Bienaimé Caventou (Pelletier & Caventou, 1820). This purified form is crystalline, white, odorless, and bitter in taste. Other salt forms of quinine, such as quinine bisulfate, quinine hydrochloride, and quinine dihydrochloride are also used as a cure and prophylaxis for malaria (Dobson, 2001; Nair, 2010).

In 1944, the American chemists **Woodward and Doering** were capable to produce synthetic quinine (Woodward & Doering, 1945). More recently, synthetic antimalarial drugs, such as chloroquine, were developed as an effective and inexpensive alternative for quinine. Certain *P. falciparum* strains became resistant against chloroquine and other synthetic drugs. Nowadays, expensive combination therapies are recommended by the WHO as a prophylaxis and treatment against malaria (Gal, 2006; WHO, 2019). More recently, the importance of quinine in the treatment of malaria increased due to the limited availability of artemisinin combination therapies (ACT) (Achan et al., 2011). Moreover, quinine is the only drug with no resistance in *P. falciparum*, although there is a small decrease in susceptibility in parts of South America and Southeast Asia. Quinine is still used as the first in-line drug for uncomplicated *P. falciparum* malaria in the first trimester of pregnancy and as part of the second-line treatments. Because of its poor tolerance, quinine is not used as a first in-line drug anymore in endemic areas (Hoffman, Campbell, & White, 2011).
Quinine has a low therapeutic index and can cause several side effects at low dosages (Achan et al., 2011). The use of quinine and its salts can cause cynchonism, which is characterized by tinnitus, dizziness, headache, dysphoria, disturbed vision, nausea, abdominal pain, hypoglycemia, hypotension, and arrythmias. If quinine is administered too quickly through an intravenous injection, it can cause heart block, ventricular fibrillation, and death (W. R. J. Taylor & White, 2004; WHO, 2015).
The increase in malaria incidence in Asia and Africa contributes to an increase in the demand for quinine and alkaloids with antimalarial properties found in the *Cinchona* bark. Despite

the abundance of *Cinchona* plantations in DR Congo, the country still has the second-highest amount of deaths due to malaria. Many Congolese citizens cannot afford the $2 cure that consists out of 21 pills or are too late to be diagnosed with the disease (Economist, 2019).
Quinine has also been used as a chemical substance to induce abortion (Morton, 1977). Quinidine, a diastereomer of quinine, has been used for cardiac arrythmias (Mason & Hondeghem, 1984). It was mainly produced by the catalytic conversion of quinine into quinidine (Nair, 2010).

3.2 Other uses of *Cinchona*

Grounded *Cinchona* bark mixed with water was recommended to be taken daily as a general prophylactic. In order to hide the bitter taste of the alkaloids, water was replaced with alcohol, resulting in the first gin-tonic (Rudo, Zeller, Siehl, Berger, & Sicker, 2018). The bitter flavor of the alkaloids found in the *Cinchona* bark, mainly caused by quinine hydrochloride and quinine sulfate, are also used nowadays in tonic water, bitter lemon, vermouth, cocktails, and liquors (Izawa et al., 2010). Nevertheless, the quinine concentration in the beverages is too low to have any therapeutical effect (Rudo et al., 2018).
The wood of the *Cinchona* trees is also used for other purposes such as fire wood and as supporting beams in houses (Hunter, 1988).

Chapter 4

DNA-based techniques as a useful tool for identification and taxonomy

DNA and protein sequences are used to retrieve the species identity of unknown samples for practical, commercial, and scientific applications. This is also called **DNA barcoding**.
Information from a sequenced genome has practical applications in selective breeding, as it is possible to control the genetic improvement of crops with much more precision and even to genetically engineer specific traits by adding, removing, or altering individual genes (Waddell, Pool, & Council, 2002). Several agricultural companies use sequencing data to characterize crop plants and drug discovery firms use sequencing data in many stages of drug development. In addition, DNA sequencing has revolutionized biological and medical research. The identification of sequence elements, which are involved in a cluster of human diseases or other phenotypes, generates a considerable amount of information on every single person, as a basis of a truly personalized medicine of the future (Lehrach, 2013).
Nowadays, DNA-based techniques are used as an advantageous complementary tool to the morphological-based taxonomy. Variable methods are available to do so and any part of the genome provides some information for taxonomic affiliation. Yet some regions are more useful than others. In particular, DNA regions (i.e. loci) which are known to be highly variable between species are used to distinguish species, because these variable loci have a larger potential to obtain diagnosis (Newmaster, Fazekas, & Ragupathy, 2006). Ribosomal DNA (rDNA) regions, in particular those of the large subunit ribosomal RNA (rRNA), are fast evolving. They are embedded between regions that are highly conserved between species (Tautz, Arctander, Minelli, Thomas, & Vogler, 2003). Other interesting barcode regions are parts of mitochondrial DNA (mtDNA) or chloroplast DNA (cpDNA). Protein sequences can also be used, but these are typically less variable. To infer relationships between individuals within a species, we can look for single nucleotide polymorphisms (SNPs) (Van Damme E, 2017).

4.1 DNA barcoding

Identifying appropriate DNA barcoding loci is difficult in land plants for several reasons. Land plants are evolutionary very divergent since their emergence about 475 million years ago, have a tendency to do large- and small-scale rearrangements in the nuclear genome, to hybridize easily, and to display different breeding behaviour (Wellman, Osterloff, & Mohiuddin, 2003). Important criteria for suitable DNA barcoding loci are:

1. They can routinely be amplified.
2. They can be sequenced easily.
3. They contain sufficient variation so that sister species can be distinguished, but also that there is sufficient sequence consistency within species so that intraspecific variation does not disturb the allocation of species.
4. Non-targeted amplification is limited.
5. They are easily annotated so that the quality of the sequences can be critically evaluated and errors can be detected (Ford et al., 2009).

The mitochondrial cytochrome *c* oxidase subunit 1-gene (*CO1*) is a protein-coding gene and present in high-copy numbers per cell. It is used to distinguish many animal species by barcoding, as this region contains adequate variability to identify species. Therefore, it has been appointed as the most appropriate barcode region for the animal kingdom (Hebert, Ratnasingham, & De Waard, 2003). This DNA barcode is haploid, maternally inherited and located on a single locus that has high discriminatory power (Fazekas et al., 2009). However, this barcoding region is not suitable to distinguish plants, as the nucleotide substitution rate is low in the *CO1* gene as well as in other regions of the plant mitochondrial genome (Fazekas et al., 2008). The quest for finding a plant equivalent has been challenging and multiple markers are necessary to classify species (Hollingsworth, Graham, & Little, 2011).

Plastid region

The chloroplast genome is usually very stable (Dong, Liu, Yu, Wang, & Zhou, 2012). The gene order and gene content is usually highly conserved and the function of several genes is known for a number of land plants (Ford et al., 2009). The chloroplast genome has a lower rate of evolution and is maternally inherited, as plastids get destroyed in pollen cells (Li & Zheng, 2018). Other benefits of the plastid genome for sequencing and DNA barcoding are the relatively small size and the high copy number per cell compared to the nuclear genome, which makes it possible to

use degraded sample material (Ford et al., 2009; Yuan et al., 2018). For these reasons, chloroplast sequences are frequently used nowadays in plant molecular systematics and DNA barcoding (Shaw et al., 2005).

Apart from coding loci, non-coding loci are also used as DNA markers. Non-coding regions show the highest rate of mutations (Palmer, Jansen, Michaels, Chase, & Manhart, 1988; Selander, Clark, & Whittam, 1991). By using these non-coding regions, a higher resolution of cpDNA can be obtained for evolutionary studies as well as for intraspecific DNA markers (Taberlet, Gielly, Pautou, & Bouvet, 1991).

Frequently used chloroplast markers are *matK* and *rbcL*. The *matK* gene (*maturase K*) is located within the intron of the chloroplast gene *trnK* (Figure 4.1). The protein it encodes is involved in the splicing of Group II introns (Hilu & Liang, 1997). The *rbcL* gene (*ribulose-bisphosphate carboxylase* gene) encodes an enzyme that is involved in the first major step of carbon fixation (Hanson & Tabita, 2001). The two plastid coding regions, *matK* and *rbcL*, need to be combined with additional markers to obtain a high resolution for species discrimination (Group et al., 2009). The substitution rate in *matK* is one of the highest in the plastid genome (Hilu & Liang, 1997). Because of the high discriminatory power of *matK* and the simple retrieval of *rbcL*, this set of markers was chosen as the core marker set for plants and assumed as the closest to the *CO1* animal analogue. Although the *matK* region is more difficult to amplify, it has a much higher discriminatory power. The *rbcL* marker only has modest discriminatory power (Hollingsworth et al., 2011).

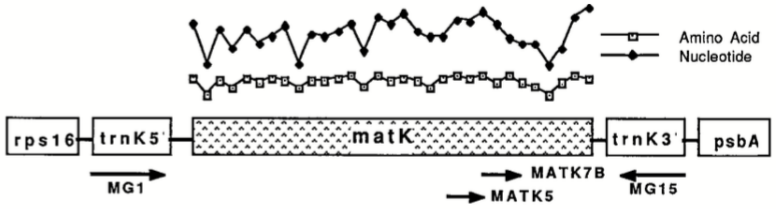


Figure 4.1: The *matK* gene and the relative position of the primers that are used for DNA amplification and sequencing. Boxed areas represent coding regions and connecting lines are spacer regions (Hilu & Liang, 1997).

The *trnL-F* region of the chloroplast genome of land plants comprises the transfer RNA genes $trnL_{\text{uaa}}$ and $trnF_{\text{gaa}}$ arranged in tandem and separated by noncoding spacer regions (Figure 4.2) (Yulita, 2013). The *trnL-F* region, including the *trnL* intron and the *trnL-F* intergenic spacer, has been shown to be a useful region for DNA barcoding within the Rubiaceae and it has widely been used for plant systematics and phylogeography since 1990 (Taberlet et al., 1991; Andersson & Antonelli, 2005). This chloroplast region is known for its potential as species-specific marker because of the low intra- and higher interspecific variability (Taberlet et al., 1991). Another

useful region to look for nucleotide sequence variation is the *rps16* intron (Oxelman, Lidén, & Berglund, 1997; Andersson & Rova, 1999; Andersson, 2002). The *rps16* (*ribosomal protein S16*) intron has been successfully used to reveal phylogenetic relationships among genera in Rubiaceae (Andersson & Rova, 1999). However, the number of informative characters in *rps16* is believed to be low, limiting its application to elucidate phylogenetic relationships between congeneric species (Shaw et al., 2005).
Another important advantage of the application of *rps16* and *trnL-F* in DNA barcoding is that an appropriate amount of sequences is archived in genomic databases such as GenBank (Andersson & Antonelli, 2005).

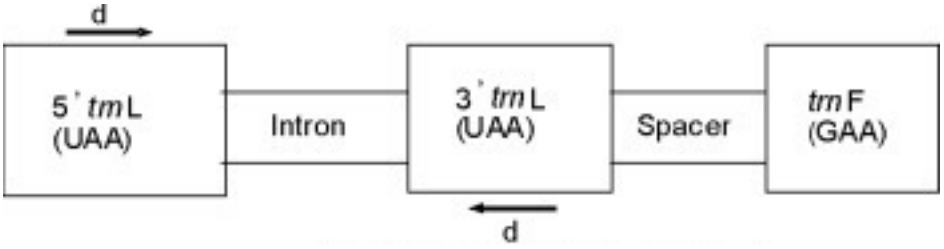


Figure 4.2: Diagram of *trnL-F* region and the relative position of the primer sequences for the intron *trnL* (d) (after (Yulita, 2013)).

ITS region

The internal transcribed spacer (ITS) region from nuclear ribosomal DNA is a frequently used barcode marker. It is transcribed with the ribosomal genes, but then spliced out. At low taxonomic levels, it has been shown that the nuclear ribosomal DNA (nrDNA) ITS region has a higher discriminatory power in several plant groups in comparison to chloroplast regions (Hollingsworth et al., 2011). The ITS region has a high substitution frequency and a substantial amount of insertions or deletions, which may complicate alignments with distantly related accessions (Andersson & Antonelli, 2005). The use of ITS as a marker has its concerns, such as the relatively high diversity between copies of ITS sequences within one individual, implying incomplete concerted evolution (Xu, Zeng, Gao, Jin, & Zhang, 2017). Moreover, different amplification protocols can result in different species assignments or in fungal contamination (Möller, 2000; Álvarez & Wendel, 2003; C. D. Bailey, Carr, Harris, & Hughes, 2003). Despite the aforementioned difficulties and concerns, the ITS region has proven to be a useful component of a plant DNA barcode (Hollingsworth et al., 2011).

The complete plastid genome and single copy or low copy nuclear genes are other DNA regions that are used for barcoding (Hollingsworth et al., 2011). Multiple loci within the plastid and nuclear genome have been used to assess relationships within the Rubiaceae tribe Cinchoneae. The choice of markers depends on the purpose of the research. The combination

of relatively fast-evolving sequences with more conservative regions can be used to achieve maximal resolution within the tribe and to place the tribe in a broader family context (Andersson & Antonelli, 2005). Apart from substitutions, there is also a relatively high frequency of insertions and deletions. In this perspective, the size of the polymerase chain reaction (PCR) products can be a useful marker to distinguish species using agarose gel electrophoresis (Taberlet et al., 1991).

4.2 Research tools and techniques available for molecular marker analysis

Plant material is usually preserved in silica gel in order to dry the plant tissue for DNA extractions, as this is one of the most cost-effective long-term storage options. Once the plant material is dry, there is a variety of storage options used by different laboratories (Hollingsworth et al., 2011).

A plethora of protocols and guidelines for DNA extraction, PCR, and DNA sequencing are available. These protocols differ in their ability to extract or amplify DNA from species of a certain taxonomic group or from samples with highly degraded DNA. Therefore, protocols must be chosen in function of the taxon sampling and sample quality (Hollingsworth et al., 2011).

Sequencing techniques

Sanger sequencing

The Sanger's chain termination technique, also called the dideoxy technique, enables the sequencing of a single DNA fragment at a time. Four reactions are run in parallel with individual fluorolabelled ddNTPs, which are chemical analogues of the dNTPs lacking the 3' hydroxyl group that is essential for chain elongation. The fluorolabelled ddNTPs get randomly incorporated, thereby impeding further elongation. Subsequently, capillary gel electrophoresis is performed, where each fragment is illuminated by a laser, which allows each attached dye to be detected. The original sequence can be ascertained by the detector, that registers the colors of the nucleotides one at a time. The recorded data are visualized in a chromatogram, which consists of a series of peaks that visualize the fluorescence intensity (Figure 4.3) (Sanger, Nicklen, & Coulson, 1977).

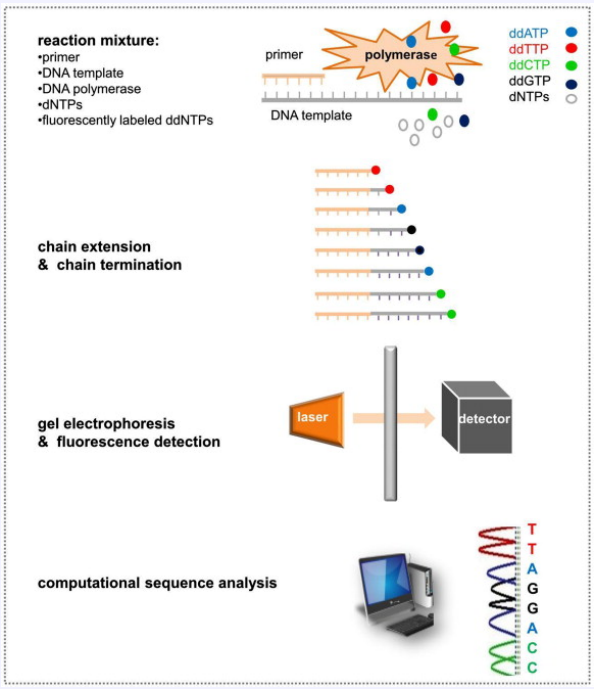


Figure 4.3: Sanger sequencing method (Zhou & Li, 2015).

High-Throughput Sequencing (HTS)

HTS is an umbrella term comprising different techniques that can sequence a large amount of DNA fragments in parallel. HTS can be used for the sequencing of complete genomes via **whole genome sequencing (WGS)**. However, because whole genome sequencing is very expensive and sequence information of the complete genome is not always needed for evolutionary analyses, HTS is often constrained to certain parts of the genome. Reduced representation sequencing (RRS) techniques, such as Genotyping-by-Sequencing (GBS), are used to obtain sequence information for a large set of short loci located throughout the genome (Dodsworth, 2015). Genome skimming is a complementary method to RRS, as a limited number of large high copy nuclear loci and plastid genomes are completely sequenced (Malé et al., 2014; Dodsworth, 2015). This methodology has been successfully applied in several cases for taxonomic affiliation (Kane et al., 2012; Besnard et al., 2013; McPherson et al., 2013; Malé et al., 2014). An additional advantage of genome skimming is that this methodology can also be applied on degraded DNA samples (Staats et al., 2013). In the future, it might be possible to sequence samples directly in the field. Oxford Nanopore Technologies (ONT) show promising advances to achieve this goal (Laver et al., 2015).

4.3 Conclusion: genomic data of *Cinchona* as a valuable tool for species identification and genetic diversity assessments

This literature survey confirmed the limited amount of data about the current natural distribution of our species of interest, *Cinchona pubescens*. Moreover, little is known about its genetic diversity within a geographic area in South America.
Maldonado et al. (2017) created genetic profiles of twenty-two *Cinchona calisaya* specimens sampled in the Yungas region of Bolivia, using four chloroplast regions (*matK*, *rps16*, *trnL-F*, and *ndhF*) and one nuclear DNA region (ITS) from leaf samples. They detected a significant phylogenetic signal in the production of two important *Cinchona* alkaloids (quinine and cinchonidine) and in the total alkaloid content (Maldonado et al., 2017). A more recent study (Cueva-Agila et al., 2019) assessed the genetic diversity in fragmented *C. officinalis* populations in the Loja province in southern Ecuador. They determined the phylogeographic distribution regarding the complex topography of the region and pinpointed the critical populations for conservation by using five nuclear microsatellite loci and the chloroplast *rps16* intron (Cueva-Agila et al., 2019). However, both studies analysed genetic diversity at a regional level.
Difficulties regarding species differentiation based on morphological characteristics and the confusing nomenclature within the genus *Cinchona* will remain due to the decrease in botanists specialised in the morphology-based taxonomy of *Cinchona* and the enduring discrepancy between botanists (L. H. Bailey & Bailey, 1976). The use of genomic data for species identification provides an additional valuable tool to the morphology-based taxonomy, as well as the solution to several biodiversity questions (Tautz et al., 2003).
Regarding the importance of *Cinchona* species, further studies that look at both the environmental and genetic variability need to be performed to understand the genotypic variation in plant biochemistry. In addition, the genetic diversity in the native range of *Cinchona* species can be assessed to identify priority populations for conservation and to select desired accessions for selective breeding. The technological and economic advances of HTS enables the application of these techniques for new research questions in plant genetics and breeding. The major goal is the detection of the link between the genotype and phenotype. This information can be used in marker-assisted breeding programs to select plants with desirable qualities based on their genotype (Poland & Rife, 2012).

Part III

Experimental

Chapter 5

Hypothese and Aims

A molecular phylogeny of Cinchona spp. over a geographical distribution in Peru and DR Congo that demonstrates a link with the alkaloid production.

Aim 1

Revealing phylogenetic patterns in *Cinchona* spp. across a broad geographic area in its region of origin, where a high genetic variability is expected among populations.

Aim 2

Identification of priority *Cinchona* populations for conservation in its native range by assessing the genetic diversity within and between populations.

Aim 3

Identifying the region of origin of cultivated *Cinchona* accessions in DR Congo and revealing phylogenetic patterns compared to its native range.

Aim 4

The detection of a phylogenetic signal in the alkaloid production.

Chapter 6

Materials and methods

6.1 Study Regions and populations

6.1.1 Central and Northern Peru

In total, 39 trees were sampled in August and September 2019 at 5 different locations in Northern and Central Peru (4 in San Ramón, 15 in Tingo María, 2 in Jaén, 10 in Bongara, and 8 in Rodríguez de Mendoza) (Figure 6.1, Table 6.1). The sampling regions were selected based on a preliminary literature study, a database survey (GBIF), and interviews with local experts (Appendix A) (Campos Ruiz, 2014).

In the sampled regions, *C. pubescens* trees mainly grow isolated or in clusters of a few trees due to over-exploitation of *Cinchona* and deforestation. During the sample collection, species identification was based on morphological characteristics. We relied on the knowledge of a local botanist and we assumed that all collected species belonged to the genus *Cinchona*, and we focused our sample collection on *Cinchona pubescens*. Samples were collected under a general permit (Resolución de Dirección General N°506_2019_MINAGRI_SERFOR_DGGSPFFS) issued on the 29th of octobre 2019 by the Peruvian Ministry of Agriculture and Irrigation, SERFOR.

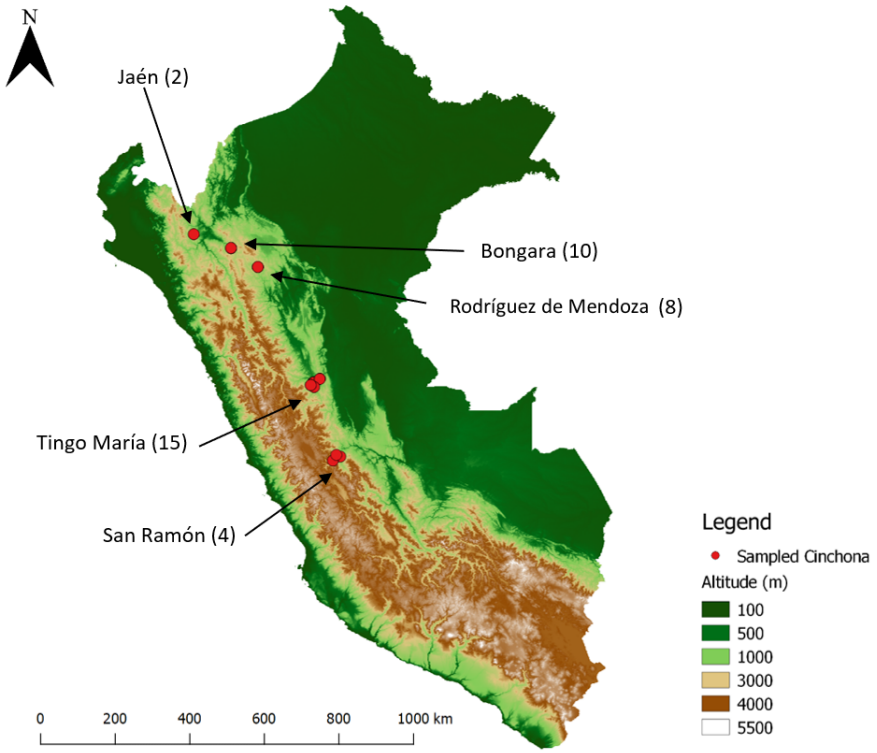


Figure 6.1: Sampled regions in Central and Northern Peru (San Ramón, Tingo María, Jaén, Bongara, and Rodríguez de Mendoza). The number of trees in each region is indicated between brackets. In total, 39 trees were sampled.

6.1.2 DR Congo

In September and October 2019, a total of 125 plantations were sampled in the highlands of South Kivu, DR Congo (74 in Walungu territory, 4 in Kabare territory, and 47 in Idjwi territory) by Prof. Landry CIZUNGU NTABOBA (Figure 6.2, Table 6.2). 3 trees per plantation were sampled. For the analysis, a sub-sample of 20 trees from different plantations was selected at random. In addition, seeds were donated to professor Luc Duchateau by the company Pharmakina during a field trip to Bukavu, DR Congo. These seeds were grown in the greenhouse of the KU Leuven. As a preliminary data exploration, the DNA of these young seedlings was analysed and added to this study. No additional geographic, environmental, or genetic information was available for these accessions.

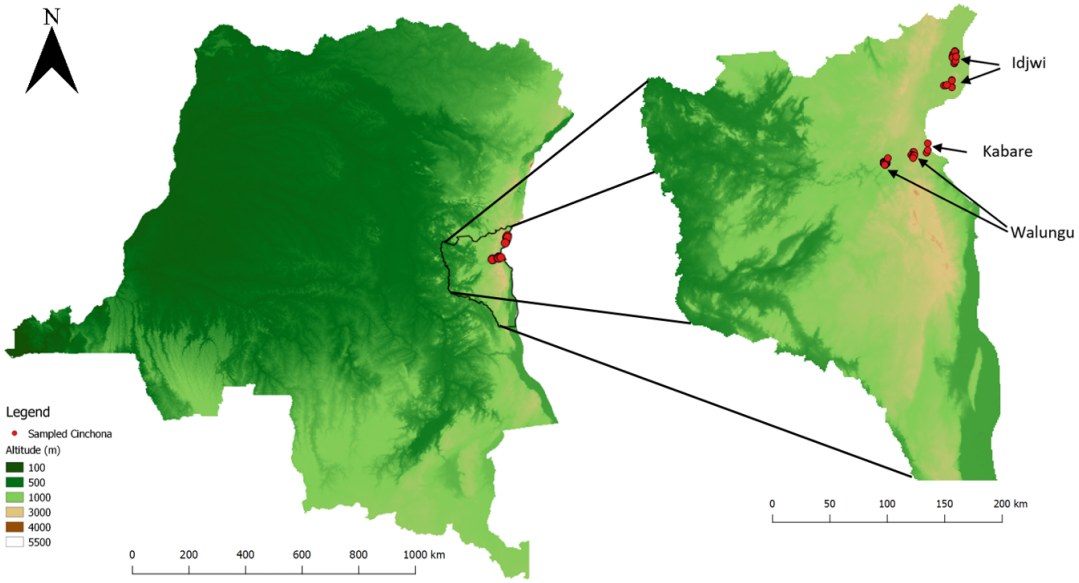


Figure 6.2: Sampled regions in South Kivu, DR Congo (Walungu territory, Kabare territory, and Idjwi territory). In total, 125 plantations were sampled, where 3 individual tree samples were taken per plantation (Table 6.2).

6.2 Data collection of *Cinchona* species

6.2.1 Central and Northern Peru

The geographic location of the sampled trees was determined by UTM coordinates with an etrex 10 Garmin GPS. Every sampled tree obtained an identification code, depending on its location, and a number according to the sampling order within one location (San Ramón: SAR, Tingo María: TIM, Bongara: BON, Rodríguez de Mendoza: MEN, Jaén: JAE).

6.2.2 Plantations of DR Congo

The geographic location of the sampled trees was determined for every plantation by Prof. Landry CIZUNGU NTABOBA. Every sample obtained a voucher code. The first number refers to the plantation, and P1, P2, or P3 refers to the sampled tree from the respective plantation that was analysed.
The 3 samples collected in Bukavu by professor Luc Duchateau were seeds from one single plantation of Pharmakina.

Table 6.1: Overview of the sampled *Cinchona* specimens (39) collected for this study in Central and Northern Peru. From left to right, the collectors of the specimens, the voucher code, the province where these specimens were collected, the elevation (m), the longitude and latitude coordinates, the collection data, the Diameter at Breast Height (DBH) (cm), and the height (m) of the sampled tree.

Collectors	Voucher	Province	Elevation (m)	Longitude	Latitude	Collection date	DBH (cm)	Height (m)
Britta and Maïa	SAR1	San Ramón	996	-75°20'1.526"	-11°8'17.981"	21/08/2019	41.2	19
Britta and Maïa	SAR2	San Ramón	1984	-75°31'3.141"	-11°14'14.093"	21/08/2019	2.7	2.8
Britta and Maïa	SAR3	San Ramón	1984	-75°31'3.141"	-11°14'14.093"	21/08/2019	2	2.2
Britta and Maïa	SAR4	San Ramón	2009	-75°25'57.595"	-11°5'47.372"	22/08/2019	18.5	9
Britta and Maïa	TIM1	Tingo María	747	-76°0'45.149"	-9°19'5.621"	26/08/2019	54	8
Britta and Maïa	TIM2	Tingo María	749	-76°0'44.394"	-9°19'5.395"	26/08/2019	20.6	10
Britta and Maïa	TIM3	Tingo María	680	-75°59'41.544"	-9°18'36.466"	27/08/2019	21	14
Britta and Maïa	TIM4	Tingo María	691	-75°59'41.643"	-9°18'36.857"	27/08/2019	59.2	13
Britta and Maïa	TIM5	Tingo María	763	-75°59'26.516"	-9°18'54.024"	27/08/2019	12	8
Britta and Maïa	TIM6	Tingo María	1412	-75°59'26.516"	-9°18'54.024"	28/08/2019	47.5	15
Britta and Maïa	TIM7	Tingo María	1409	-75°50'11.165"	-9°13'22.634"	28/08/2019	7.1	8
Britta and Maïa	TIM8	Tingo María	1299	-75°50'21.958"	-9°13'41.363"	28/08/2019	29.5	10
Britta and Maïa	TIM9	Tingo María	840	-75°58'47.672"	-9°25'30.514"	30/08/2019	34.2	15
Britta and Maïa	TIM10	Tingo María	845	-75°58'47.411"	-9°25'30.808"	30/08/2019	25	14
Britta and Maïa	TIM11	Tingo María	845	-75°58'47.411"	-9°25'30.71"	30/08/2019	37.4	12
Britta and Maïa	TIM12	Tingo María	1215	-76°3'41.166"	-9°22'43.822"	31/08/2019	23.5	13
Britta and Maïa	TIM13	Tingo María	1215	-76°3'41.264"	-9°22'43.79"	31/08/2019	24.7	14,5
Britta and Maïa	TIM14	Tingo María	1209	-76°3'42.018"	-9°22'43.657"	31/08/2019	20.8	10
Britta and Maïa	TIM15	Tingo María	1210	-76°3'41.986"	-9°22'43.918"	31/08/2019	21.3	12
Britta and Maïa	JAE1	Jaén	2298	-78°56'25.361"	-5°39'53.869"	05/09/2019	25.8	13
Britta and Maïa	JAE2	Jaén	2301	-78°56'25.103"	-5°39'53.25"	05/09/2019	20.7	8
Britta and Maïa	BON2	Bongara	2618	-78°1'21.733"	-6°0'34.786"	10/09/2019	4.9	6
Britta and Maïa	BON3	Bongara	2628	-78°1'21.731"	-6°0'35.176"	10/09/2019	8.7	9
Britta and Maïa	BON4	Bongara	2628	-78°1'22.214"	-6°0'36.057"	10/09/2019	8.4	8
Britta and Maïa	BON5	Bongara	2656	-78°1'21.5"	-6°0'35.826"	10/09/2019	9.1	9
Britta and Maïa	BON6	Bongara	2604	-78°1'19.664"	-6°0'15.001"	13/09/2019	16.3	5
Britta and Maïa	BON7	Bongara	2622	-78°1'21.094"	-6°0'14.943"	13/09/2019	12.2	7
Britta and Maïa	BON8	Bongara	2604	-78°1'19.664"	-6°0'15.001"	13/09/2019	17.8	7
Britta and Maïa	BON9	Bongara	2608	-78°1'19.196"	-6°0'17.47"	13/09/2019	35.3	8
Britta and Maïa	BON10	Bongara	2623	-78°1'22.833"	-6°0'17.815"	13/09/2019	37.8	7
Britta and Maïa	BON11	Bongara	2682	-78°1'26.327"	-6°0'14.386"	13/09/2019	33.6	6.5
Britta and Maïa	MEN1	Rodríguez de Mendoza	1386	-77°21'48.73"	-6°28'20.341"	11/09/2019	10.1	8
Britta and Maïa	MEN2	Rodríguez de Mendoza	1386	-77°21'48.73"	-6°28'20.341"	11/09/2019	15.1	11
Britta and Maïa	MEN3	Rodríguez de Mendoza	1386	-77°21'48.73"	-6°28'20.341"	11/09/2019	11.2	9
Britta and Maïa	MEN4	Rodríguez de Mendoza	1386	-77°21'48.73"	-6°28'20.341"	11/09/2019	10.6	8
Britta and Maïa	MEN5	Rodríguez de Mendoza	1386	-77°21'48.73"	-6°28'20.341"	12/09/2019	11.9	9
Britta and Maïa	MEN6	Rodríguez de Mendoza	1384	-77°21'48.303"	-6°28'19.464"	12/09/2019	10.8	8
Britta and Maïa	MEN7	Rodríguez de Mendoza	1369	-77°21'48.481"	-6°28'15.786"	12/09/2019	11.8	9
Britta and Maïa	MEN8	Rodríguez de Mendoza	1375	-77°21'48.118"	-6°28'14.649"	12/09/2019	14.2	9

Table 6.2: Summary of the sampled *Cinchona* specimens (23) for this study in DR Congo. Data about their collectors, voucher code, sampling location, elevation (*m*), longitude and latitude coordinates, and collection date are provided. A dash ("-") indicates unknown data.

Collectors	Voucher	Territory	City	Elevation (*m*)	Longitude	Latitude	Collection date
Landry and colleagues	1P2	Walungu	Bwiralike	1426	28°34'53.364"	-2°43'59.844"	17/09/2019
Landry and colleagues	4P1	Walungu	Luzirhu	1437	28°33'36.504"	-2°44'5.064"	18/09/2019
Landry and colleagues	9P1	Walungu	Luzirhu	1433	2833'52.236"	-2°44'24.108"	18/09/2019
Landry and colleagues	16P3	Walungu	Luzirhu	1452	28°33'53.856"	-2°44'41.208"	18/09/2019
Landry and colleagues	19P2	Walungu	Kaliba	1432	28°34'18.804"	-2°44'39.912"	18/09/2019
Landry and colleagues	23P1	Walungu	Kalangwe	1438	28°35'2.328"	-2°44'25.44"	19/09/2019
Landry and colleagues	30	Walungu	Kalangwe	1453	28°34'47.712"	-2°45'3.276"	19/09/2019
Landry and colleagues	35P3	Walungu	Kalangwe	1420	28°34'38.424"	-2°44'55.788"	19/09/2019
Landry and colleagues	40P3	Walungu	Kaliba	1412	28°34'12.108"	-2°45'27.468"	19/09/2019
Landry and colleagues	47P1	Walungu	Kalangwe	1869	28°46'27.84"	-2°39'51.876"	23/09/2019
Landry and colleagues	54P2	Idjwi	Ikumba	1861	28°45'33.12"	-2°41'14.064"	23/09/2019
Landry and colleagues	63P2	Idjwi	Burherha	1858	28°46'17.58"	-2°41'30.48"	24/09/2019
Landry and colleagues	70P2	Idjwi	Burherha	1863	28°46'28.164"	-2°41'34.26"	24/09/2019
Landry and colleagues	75P3	Kabare	Nshmbi	1584	28°51'55.548"	-2°40'6.672"	28/09/2019
Landry and colleagues	80P3	Idjwi	Bugarula	1461	29°3'48.708"	-2°2'11.292"	03/10/2019
Landry and colleagues	83P2	Idjwi	Bushusha	1575	29°2'59.172"	-1°59'21.228"	03/10/2019
Landry and colleagues	95P1	Idjwi	Buyumbu	1646	29°4'5.736"	-1°57'11.124"	04/10/2019
Landry and colleagues	100P2	Idjwi	Buyumbu	1618	29°4'13.044"	-1°57'13.428"	04/10/2019
Landry and colleagues	111P2	Idjwi	Muganzo	1791	29°4'38.172"	-1°59'16.62"	04/10/2019
Landry and colleagues	125P3	Idjwi	Ngonda	1533	29°0'32.688"	-2°11'30.012"	06/10/2019
Luc Duchateau	N32	South Kivu	Bukavu	-	-	-	-
Luc Duchateau	BV1	South Kivu	Bukavu	-	-	-	-
Luc Duchateau	K5	South Kivu	Bukavu	-	-	-	-

6.2.3 Morphological characterization of the *Cinchona* trees and environmental factors

Tree dimensions

For each individual tree, the total height (*m*) and the DBH (*cm*) were determined. Tree diameter was measured including the bark at 1.3 *m* above the ground, except for particular cases (Figure 6.3). Measurements were performed using diameter tape (*cm*). Tree height was measured by goniometrical proportions. A crown description was performed based on field observation. The crown form was classified by means of a Crown Form (CF) score developed by Dawkins (Dawkins, 1963). For each individual tree, photos were taken of the morphological characteristics (e.g. fruits, leaves, seeds, flowers, and trunk) (Figure 6.4).

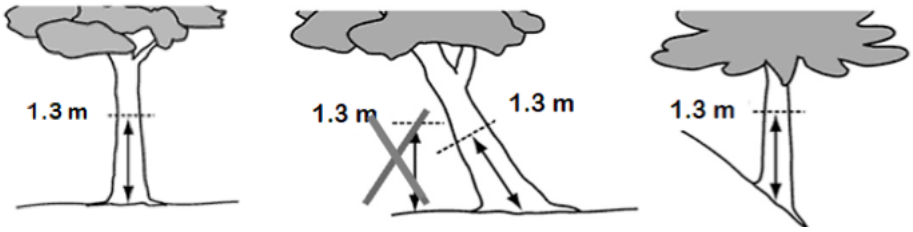
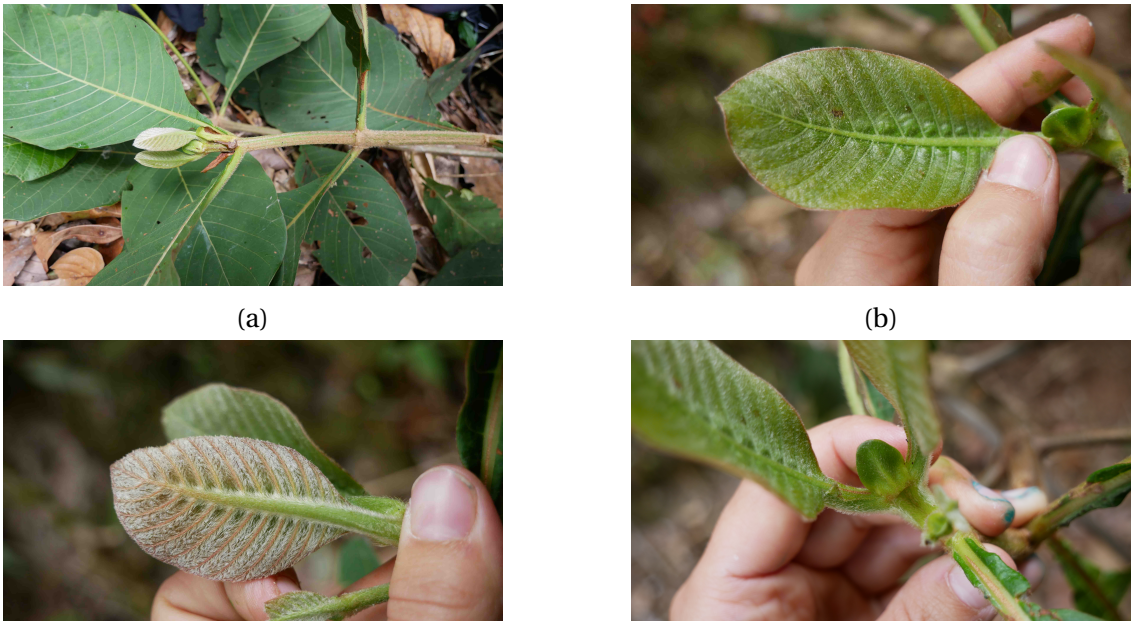


Figure 6.3: Measuring Diameter at Breast Height (cm) on different slopes and tree angles.



(a) (b)

(c) (d)

Figure 6.4: *Cinchona pubescens*: (a) and (d) opposite leaves, (b) veined leaf, and (c) hairy morphotype on the abaxial leaf surface.

Environmental conditions

Canopy cover, which is the area covered by the trunk, branches, and leaves of a tree, was estimated for each sampled tree. A first estimation was done by observation of the ground coverage (in %). The canopy was divided into two levels. A ground coverage estimation was performed as observed from the ground. A second estimation of the total ground coverage was carried out to assess the effect of the upper part of the canopy if applicable (above 7 m). The height of the tree determined which estimation was the most relevant to evaluate the effect of the canopy on the *Cinchona* tree. Orthogonal cover photos were taken at each cardinal point (North, South, West, and East) in a 4 m radius ring around each *Cinchona* tree (Figure 6.5). In addition, the vegetation associated with the *Cinchona* trees was characterised. These results were further analysed by Maïa De Ridder (De Ridder, 2020).

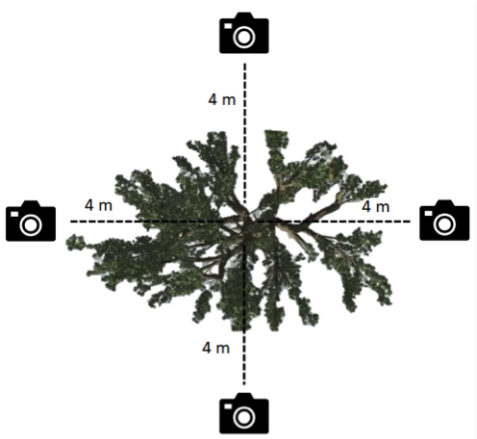


Figure 6.5: Orthogonal photography view from above.

Climate data

Climatological data were taken from the WorldClim database and extracted in QGIS 2.18.17 using the GPS coordinates (QGIS, 2009). The mean annual temperature and precipitation data with a resolution of 30 s were taken for every location in Peru and DR Congo for the period 1960-2018.

6.2.4 Sampling

Bark

Bark samples were taken using a hammer and a chisel. An area of 2x2 cm^2 with a depth of 0.5-2.5 cm was sampled, as this is sufficient to analyse the alkaloid concentration while minimizing the damage to the tree. Subsequently, natural honey was applied to the wound to avoid infection and assure fast regeneration.
Three bark samples were collected at different heights (30 cm, 1.3 m, and 2.3 m) of the trunk for chemical analysis to account for possible variation in concentrations of chemical compounds along the tree (Nair, 2010). Trees below 2.3 m were only sampled at available heights.
Bark samples were dried in an oven at 55°C and eventually stored in silica gel in order to avoid the re-uptake of moist. A few bark samples were covered with lichens, which were brushed off before the bark samples were ground to homogeneity using a grinding device.

Leaves

A total of 13 leaf samples were collected for DNA (3) and nutrient (10) analysis. The leaves for nutrient analysis were stored separately in paper bags for transportation, dried at 65°C, and subsequently weighted for specific leaf area (SLA) calculation. This analysis was further performed by Maïa De Ridder (De Ridder, 2020). The leaves for DNA analysis were stored separately in paper bags in silica gel for subsequent DNA extraction and analysis.

Soil

Three soil samples were collected in a 1 m radius ring around each *Cinchona* tree at 3 different depths (0-10 cm, 10-20 cm, and 20-30 cm). For each tree, the 3 different subsamples of each depth were mixed in a bucket and stored in a labeled plastic bag in a cool environment. Subsequently, soil samples were dried in an oven at 65°C for 24 h. Soil samples were processed for final analysis and stored in cardboard boxes.

6.3 Laboratory analysis

6.3.1 Detection of alkaloids in the bark

The COVID-19 crisis prohibited the optimisation of the extraction protocol of the four major alkaloids (Figure 2.5). For this reason, alkaloids were not extracted from the bark samples and this analysis was not included in this study.

6.3.2 Nutrient analysis in the leaves and the soil

Leaf nutrient analysis was performed at the campus of Gontrode, Ghent University. The dried leaves were ground to homogeneity using a grinding device. Subsequently, analysis on C, N, P, K, Ca, and Mg-content was carried out. The C and N-concentrations in biomass were measured by high temperature combustion at 1150°C using an elemental analyzer (Vario MACRO cube CNS, Elementar, Germany). The leaf P-concentration was obtained after digesting 100 mg of the sample with 0.4 ml $HClO_4$ (65%) and 2 ml HNO_3 (70%) in Teflon bombs for 4 h at 140°C. Phosphorus is measured colorimetrically using the malachite green procedure (Lajtha & Jarrell, 1999). The K, Mg, and Ca-concentrations were obtained after digesting 100 mg of the sample with 0.4 ml $HClO_4$ (65%) and 2 ml HNO_3 (70%) in Teflon bombs for 4 h at 140°C and measured by atomic absorption spectrophotometry (AA240FS, Fast Sequential AAS). The soil nutrient analysis was performed at La Universidad Nacional Agraria La Molina in the UNALM Laboratory. K, Ca, and Mg concentrations were determined with the ammonium acetate method at pH 7, which extracted the cations with ammonium acetate and thereafter determined concentrations were measured by atomic absorption spectrophotometry. The samples were also analysed on extractable phosphorus with the modified Olsen method. Furthermore, the density was determined with a sampled cylinder, the pH was measured with a 1:1 soil to water ratio solution, and the Organic Matter content (%) was calculated with the method of Walkley and Black (Walkley & Black, 1934). The $CaCO_3$ content characterisation (%) and a mechanical analysis (% Sand, % Silt, % Clay) of these samples was also carried out.

6.3.3 DNA sequencing

Total genomic DNA was extracted from 10 mg dried leaf material using a Plant/Fungi DNA Isolation Kit (Norgen Biotek Corp, Thorold, Canada) with cell lysis performed in a standard lysis buffer according to the manufacturer's protocol. Prior to cell lysis, plant material was homogenised using zirconium beads in the Bead Rupture Elite device (Omni International, USA). The DNA concentration and the sample purity was quantified with a NanoDrop 2000 C spectrophotometer (Thermo Fisher Scientific, USA).

Four chloroplast (*trnL-F*, *matK*, *rps16*, and *ndhF*) and one nuclear (ITS) DNA region were sequenced using the primer sets listed in Table 6.3. The selection of marker regions was based on previous studies of related taxa showing useful proportions of phylogenetically informative sites within the tribe Cinchoneae (Andersson & Antonelli, 2005; Manns & Bremer, 2010). PCR amplification was performed in a 25 µL reaction mixture, containing 5 µL ALLin HiFi buffer (0.25 mM dNTPs, 3mM $MgCl_2$, and enhancers and stabilizers unspecified by the manufacturer), 0.25 µL ALLin HiFi DNA Polymerase (highQu, Germany), 17.75 µL H_2O, 0.5 µL forward primer, 0.5 µL reverse primer, and 1 µL template DNA using the PCR programs described in Table 6.3. PCR products were purified using Agencourt AMPure XP (Beckman Coulter, Inc., USA) before Sanger sequencing was performed by Macrogen (Macrogen Europe Inc., Amsterdam, the Netherlands). All products were sequenced in both forward and reverse directions, apart from *trnL-F*, which was only sequenced in the reverse direction as our test results showed that the quality of the data that were sequenced in the forward direction was too low.

Table 6.3: List of primers used in this study. Data on the DNA region, primer names, primer sequence 5'-3', reference, and thermocycling protocol are provided.

DNA region	Primer names	Sequence 5'-3'	Reference	Thermocycling protocol
ITS	18S-1830F 26S-25R	5'-AACAAGGTTTCCGTAGGTGA-3' 5'-TATGCTTAAAYTCAGCGGGT-3'	(Nickrent, Schuette, & Starr, 1994)	'Activation of the polymerase at 95°C for 1 min followed by 33 cycles of denaturation at 95°C for 15 s, annealing at 50°C for 15 s, and a final elongation at 72°C for 30 s.
trnL-F	cF fR	5'-CGAAATCGGTAGACGCTACTACG-3' 5'-ATTTGAACTGGTGACACGAG-3'	(Taberlet et al., 1991)	'Activation of the polymerase at 95°C for 1 min followed by 33 cycles of denaturation at 95°C for 15 s, annealing at 55°C for 15 s, and a final elongation at 72°C for 45 s.
rps16	rpsF rpsR2	5'-GTGGTAGAAAGCAACGTGCGACTT-3' 5'-TCGGGATCGAACATCAATTGCAAC-3'	(Oxelman et al., 1997)	'Activation of the polymerase at 95°C for 1 min followed by 33 cycles of denaturation at 95°C for 15 s, annealing at 50°C for 15 s, and a final elongation at 72°C for 30 s.
tmK-matK-tmK	matK1198f matk2053r	5'-CTGTGTTAGATATACNAATACCCC-3' 5'-TTAGCRCAAGAYAGTCGAAGTA-3'	(Andersson & Antonelli, 2005)	'Activation of the polymerase at 95°C for 1 min followed by 33 cycles of denaturation at 95°C for 15 s, annealing at 50°C for 15 s, and a final elongation at 72°C for 30 s.

6.4 Data analysis

6.4.1 Tree characterization

Histograms were created for the following tree characteristics: DBH (cm), height (m), SLA (mm^2/mg), and crown area (m^2), and density plots for the following environmental characteristics: elevation (m), precipitation (mm/year), and temperature (°C) using R *v 3.6.2*.

6.4.2 Specific Leaf Area (SLA)

The Specific Leaf Area (SLA) is the ratio of the light capturing surface of a leaf per unit of invested dry mass. This is a frequently used leaf trait and is most commonly expressed in mm^2/mg. Plants adjust their SLA to the ambient light conditions under which the leaves develop. The leaf Area of the Peruvian samples was calculated by processing the fresh leaf scans in Image-J in order to determine the surface in mm^2/mg. The leaves of the Congolese samples were not freshly scanned. The SLA of the Congolese samples was determined for one randomly selected leaf per tree (flat dried between dry sheets of paper for 3 days at 50°C) by dividing the leaf area measured with a Li-Cor Portable Area Meter Li-3100 (Li-Cor Biosciences, Lincoln, NE, USA) by the leaf dry mass. The SLA data analysis was performed by Maïa De Ridder (2020).

6.4.3 Phylogenetic analysis

Assembling a genetic dataset

Sequences were assembled and manually corrected for base-calling errors using GENEIOUS *v R9* (Kearse et al., 2012). In addition, each sequence of our own samples was analysed with the Basic Local Alignment Search Tool (BLAST) from GenBank to retrieve the species identity of the samples and to detect sample contamination. Reference sequences from *Cinchona* spp. and from *Ladenbergia* spp. were retrieved from Genbank and added to our dataset (Table 6.5 and 6.6).

Multiple Sequence Alignment

Sequences were aligned using MAFFT (Multiple Alignment using Fast Fourier Transform) as implemented in GENEIOUS. Using the same program, the alignment ends were trimmed manually to correct for variable start and stop positions in different samples.

Determination of haplotype variation

Haplotypes were delineated for each nuclear and chloroplast DNA region. A haplotype refers to a cluster of SNPs, which are variations at single positions in the DNA sequence among individuals, and indels, which are insertions and deletions (Wheeler et al., 2005). A haplotype was defined by the genotype of a sample at all SNP and indel positions in a region.

Determination of a substitution model

In order to reconstruct a phylogenetic tree, an appropriate substitution model was selected. A substitution model attempts to estimate the rate of nucleotide substitutions at a given site and the distribution of substitutions across the entire sequence. A variety of statistical substitution models are available. They are all based on certain assumptions and are supposed to take hidden genetic variation (e.g. back mutations) into account. The Jukes and Cantor model (JC) is the simplest substitution model for nucleotide sequences. It assumes that each nucleotide (A, C, T, G) occurs with the same frequency (25 %) and is substituted with an equal rate. However, transition mutations (A $\longleftrightarrow$ G, C $\longleftrightarrow$ T) often occur more frequently than transversion mutations (A $\longleftrightarrow$ C, G $\longleftrightarrow$ T). More complex models, such as the Hasegawa–Kishono–Yano (HKY) and the Felsenstein model (F81), assume that the nucleotides occur at different frequencies and that transversions and transitions occur at different rates. The general time reversible (GTR) model is even more complex. In addition to the assumption of variable frequencies for the occurence of nucleotides, it assumes different substitution rates for each pair of nucleotides. For models

with non-uniform nucleotide frequencies, nucleotide frequencies are estimated based on the observed frequenties in the alignment (Choudhuri, 2014).
Information criteria assess model uncertainty. The Akaike information criterion (AIC) is an asymptotically unbiased estimator of the Kullback-Leibler information quantity (Akaike, 1974; Kullback & Leibler, 1951). When the sample size is small in comparison to the number of parameters, the use of the corrected Akaike information criterion (AICc) is recommended (Sugiura, 1978; Hurvich & Tsai, 1989). Information criteria rank the models to select the least parameter-rich model with the highest likelihood.
Data were partitioned into nuclear (ITS) and chloroplast (*trnL-F*, *matK*, *rps16*) regions and partition-specific parameters were estimated for each region. The most appropriate substitution model was selected for each barcode region separately with the corrected Akaike information criterion (AICc) using the program jModelTest2 (Table 6.4) (Darriba, Taboada, Doallo, & Posada, 2012).

Table 6.4: Overview of the substitution models that were selected for each barcode marker (ITS, *matK*, *rps16*, and *trnL-F*). GTR: the General Time Reversible model, GTR+I: the General Time Reversible model where a proportion of the sites is invariable and HKY: the Hasegawa–Kishono–Yano model.

Region	**Model**
ITS	GTR+I
matK	HKY
rps16	HKY
trnL-F	GTR

Tree building and phylogenetic inference

Phylogenetic relationships were inferred using a standard Bayesian approach implemented in MrBAYES *v 3.2.7*, which reconstructs a phylogeny based on the posterior probability distribution of trees using a Markov Chain Monte Carlo sampling algorithm (MCMC) (Ronquist et al., 2012). 3,000,000 generations were sampled in each run using four chains and a sampling frequency of 100. Three replicate runs were accomplished in parallel and convergence between runs was evaluated using the average standard deviation of split frequencies (< 0.01). Within each run, chain convergence was assessed with the effective sampling size (ESS > 200) and the potential scale reduction factor (PSRF close to 1). Trace files were assessed in TRACER *v 1.7* to determine an appropriate burn-in phase (in this case the first 10,000 sampled trees) (Suchard et al., 2018). The tree was visualised with FigTree *v 1.4.4* (Rambaut, 2012).

Table 6.5: List of samples used for molecular analyses. "+" indicates that the sequence was available, "-" that the sequence was not available.

Code in the paper	Voucher number	ITS	*trnL-F*	*matK*	*rps16*
***Cinchona* sp. - Peru**					
Cinchona sp. SAR1	SAR1	-	+	+	+
Cinchona sp. SAR2	SAR2	+	+	+	+
Cinchona sp. SAR3	SAR3	+	+	+	+
Cinchona sp. SAR4	SAR4	+	+	+	+
Cinchona sp. TIM1	TIM1	+	+	+	+
Cinchona sp. TIM2	TIM2	+	+	+	+
Cinchona sp. TIM3	TIM3	+	+	+	+
Cinchona sp. TIM4	TIM4	+	+	+	-
Cinchona sp. TIM5	TIM5	+	+	+	+
Cinchona sp. TIM6	TIM6	+	+	+	+
Cinchona sp. TIM7	TIM7	+	+	+	+
Cinchona sp. TIM8	TIM8	+	+	+	+
Cinchona sp. TIM9	TIM9	+	+	+	+
Cinchona sp. TIM10	TIM10	+	+	+	+
Cinchona sp. TIM11	TIM11	+	+	+	+
Cinchona sp. TIM12	TIM12	+	+	+	+
Cinchona sp. TIM13	TIM13	+	+	+	+
Cinchona sp. TIM14	TIM14	+	+	+	+
Cinchona sp. TIM15	TIM15	+	+	+	+
Cinchona sp. JAE1	JAE1	+	+	+	+
Cinchona sp. JAE2	JAE2	+	+	+	+
Cinchona sp. BON2	BON2	-	-	-	-
Cinchona sp. BON3	BON3	+	+	+	+
Cinchona sp. BON4	BON4	+	+	+	+
Cinchona sp. BON5	BON5	-	-	-	-
Cinchona sp. BON6	BON6	+	+	+	+
Cinchona sp. BON7	BON7	+	+	+	+
Cinchona sp. BON8	BON8	+	-	+	+
Cinchona sp. BON9	BON9	+	+	+	+
Cinchona sp. BON10	BON10	+	+	+	+
Cinchona sp. BON11	BON11	+	-	+	+
Cinchona sp. MEN1	MEN1	+	+	+	+
Cinchona sp. MEN2	MEN2	+	+	+	+
Cinchona sp. MEN3	MEN3	+	+	+	+
Cinchona sp. MEN4	MEN4	-	-	+	+
Cinchona sp. MEN5	MEN5	+	+	+	+
Cinchona sp. MEN6	MEN6	-	+	+	+
Cinchona sp. MEN7	MEN7	+	+	+	+
Cinchona sp. MEN8	MEN8	-	+	+	+
***Cinchona* sp. - DR Congo**					
Cinchona sp. 4	N32	+	+	+	+
Cinchona sp. 5	BV1	+	+	+	+
Cinchona sp. 9	K5	+	+	+	+
Cinchona sp. 40	4P1	-	-	-	-
Cinchona sp. 41	9P1	-	-	-	-
Cinchona sp. 42	23P1	-	-	-	-
Cinchona sp. 43	47P1	-	-	+	+
Cinchona sp. 44	95P1	-	-	+	+
Cinchona sp. 45	1P2	-	-	-	-
Cinchona sp. 46	19P2	-	-	+	-
Cinchona sp. 47	70P2	+	-	+	-
Cinchona sp. 48	54P2	-	-	-	-
Cinchona sp. 49	63P2	+	-	+	+
Cinchona sp. 50	83P2	-	-	-	-
Cinchona sp. 51	100P2	-	-	+	+
Cinchona sp. 52	111P2	+	-	-	-
Cinchona sp. 53	16P3	-	-	-	+
Cinchona sp. 54	35P3	-	-	+	+
Cinchona sp. 55	40P3	+	-	+	+
Cinchona sp. 56	75P3	-	-	-	-
Cinchona sp. 57	80P3	-	-	+	+
Cinchona sp. 58	125P3	-	-	-	-
Cinchona sp. 59	30	-	-	-	-

Table 6.6: List of reference samples used for molecular analyses together with their GenBank accession numbers and voucher information. Missing data on voucher number is indicated by *.

Code in the paper	Voucher number/Citation	Genbank accession number			
		ITS	*trnL-F*	*matK*	*rps16*
Cinchona pubescens					
C. pubescens KJ8156551	*McDowell 4613* (Bremer & Manen, 2000)			KJ815655.1	
C. pubescens LN6803441	*Bremer 2733* (Wikström, Kainulainen, Razafimandimbison, Smedmark, & Bremer, 2016)			LN680344.1	
C. pubescens Z701971	* (Andersson & Antonelli, 2005)			Z70197	
C. pubescens 3694	*Stâhl 3694* (Andersson & Antonelli, 2005)	AY538356	AY538451		
Cinchona officinalis					
C. officinalis 2551	*Andersson & Nilsson 2551* (GB) (Andersson & Antonelli, 2005)	AY538354	AY538450	AY538381	AY538427
***Cinchona calisaya* WEDD.**					
C. calisaya CMG3998	*Maldonado 3998* (Maldonado et al., 2017)	KY676314	KY676254	KY676277	KY676212
C. calisaya CMG4001	*Maldonado 4001* (Maldonado et al., 2017)	KY676306	KY676266	KY676290	KY676210
C. calisaya CMG4004	*Maldonado 4004* (Maldonado et al., 2017)	KY676299	KY676255	KY676279	KY676214
C. calisaya CMG4214	*Maldonado 4214* (Maldonado et al., 2017)	KY676318	KY676245	KY676270	KY676224
C. calisaya CMG4216	*Maldonado 4216* (Maldonado et al., 2017)	KY676309	KY676259	KY676283	KY676202
C. calisaya CMG4228	*Maldonado 4228* (Maldonado et al., 2017)	KY676304	KY676251	KY676276	KY676219
***Cinchona micrantha* RUIZ & PAV.**					
C. micrantha BEV12	*Escobari 12* (Maldonado et al., 2017)	KY676294		KY676292	KY676221
C. micrantha BEV15	*Escobari 15* (Maldonado et al., 2017)	KY676295	KY676252	KY676293	KY676222
C. micrantha BEV19	*Escobari 19* (Maldonado et al., 2017)	KY676296	KY676267	KY676269	KY676223
***Ladenbergia* spp.**					
Ladenbergia oblongifolia	*Persson & Gustafsson 245* (Andersson & Antonelli, 2005)	AY538366	AY538462	AY538401	AY538436
Ladenbergia pavonii	*Knudsen 548* (Andersson & Antonelli, 2005)	AY538367	AY538463	AY538402	AY538437

6.4.4 Phylogenetic tree linked to environmental traits and tree characteristics

Subsequently, environmental traits (elevation (m) and soil pH) and tree characteristics (SLA (mm^2/mg), leaf phosphorous concentration (g/kg), and leaf C/N ratio) were linked to the phylogenetic tree in R *v 3.6.2* by using the package *treeio* and heatmap visualization (Wang et al., 2020).

6.4.5 Multivariate analysis, genetic diversity and differentiation estimation

Allelic richness (number of alleles) is a measure of genetic diversity indicative of a population's long-term potential for adaptability and persistence (Greenbaum, Templeton, Zarmi, & Bar-David, 2014). The rarefraction method was used to correct for small sample sizes and to provide unbiased estimates of the allelic richness (Leberg, 2002). Genetic diversity was quantified as the allelic richness using R *v 3.6.2* (R Core Team, 2013) and the function *allelicrichness* of the pegas package (Paradis, 2010). The genetic clustering of samples was assessed with a principal component analysis (PCA) using the *glPCA* function in the package adegenet (Jombart, 2008). Pairwise comparisons of population differentiation were performed by calculating the fixation index (F_{ST}) (Wright, 1949, 1965). F_{ST} is the proportion of the total genetic variance contained in a subpopulation (the S subscript) relative to the total genetic variance in the population (the T subscript). Values can range from 0 to 1. A high F_{ST} value implies a considerable degree of differentiation among populations. The fixation index (F_{ST}) was calculated following Weir and

Cockerham (1984) using the function *pairwise.WCfst* in the package hierfstat (Goudet, 2005). The *C. calisaya* reference accessions, which were sampled in several provinces in Bolivia (Nor Yungas, Coroico, and Chulumani), were included in the allelic richness and F_{ST} calculations in addition to the samples collected for this study from Peru and DR Congo.

Chapter 7

Results

7.1 Sampling results

Environmental characteristics were summarized in density plots (Figure 7.1). The elevation of the 125 sampled plantations in DR Congo was on average 1619.13 m (s.e. = 15.59 m), whereas the elevation of the sampled regions in Northern and Central Peru was on average 1641.8 m (s.e. = 113 m). The sampled regions in Northern and Central Peru had a mean annual temperature of 19.54 °C (s.e. = 0.61 °C) and a mean annual precipitation of 1501 $mm/year$ (s.e. = 103 $mm/year$), whereas the sampled regions in DR Congo had a mean annual temperature of 23.04 °C (s.e. = 0.17 °C) and a mean annual precipitation of 1705.12 $mm/year$ (s.e. = 16.16 $mm/year$).
The tree height of the sampled species in Northern and Central Peru varied between 2.2 and 19 m and was on average 9.6 m (s.e. = 0.56 m). The range of the DBH of the peruvian samples was between 2 and 59.2 cm, with an average of 21.32 cm (s.e. = 2.28 cm) (Figure 7.2).

7.2 Molecular study results

7.2.1 Genotyping of the collected samples

A final dataset of 37 Peruvian individuals and 14 Congolese samples that were genotyped for four markers (*matK*, *trnL-F*, *rps16*, and ITS) was used to explore population genetics and obtain phylogenetic inference. We were not able to obtain DNA sequences from 2 Peruvian and 9 Congolese samples. Sequences of the ITS and the *trnL-F* region could not be reliably generated for resp. 16 out of 23 and 20 out of 23 Congolese specimens (Table 6.5).

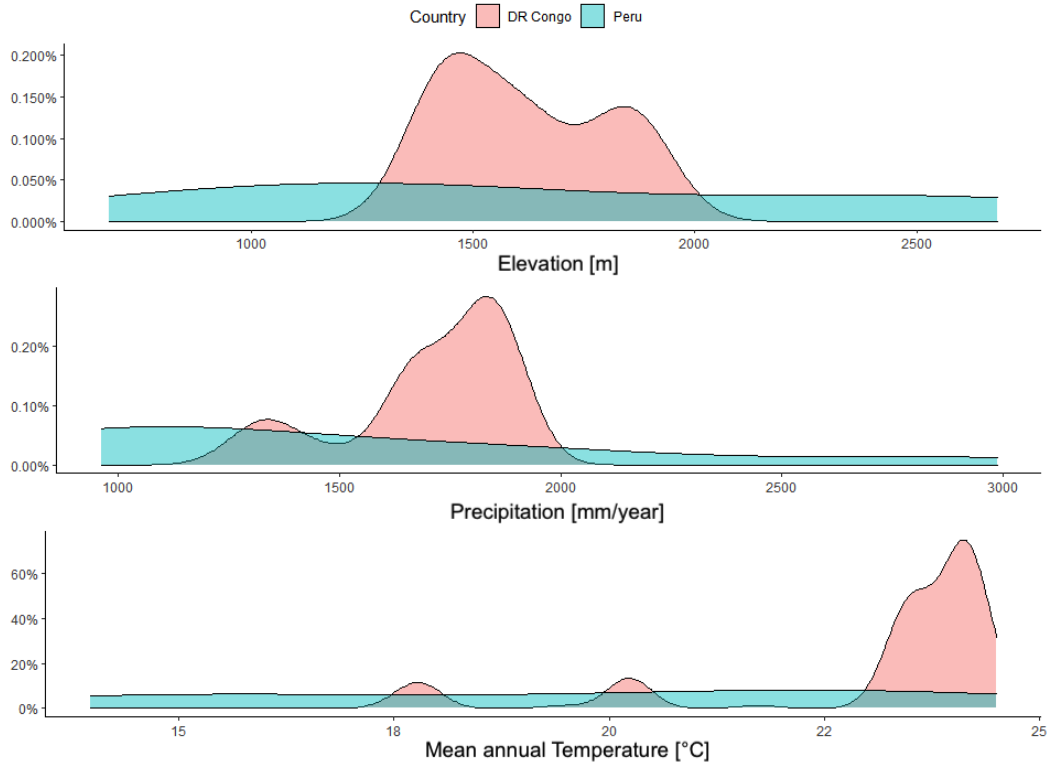


Figure 7.1: Density plots of the environmental characteristics from the sampled regions in Northern and Central Peru (blue) and the 125 sampled plantations in DR Congo (pink). Y-axis shows the density (%) and x-axis gives the range of the variable. Top: elevation (m), middle: precipitation ($mm/year$), and bottom mean annual temperature (°C).

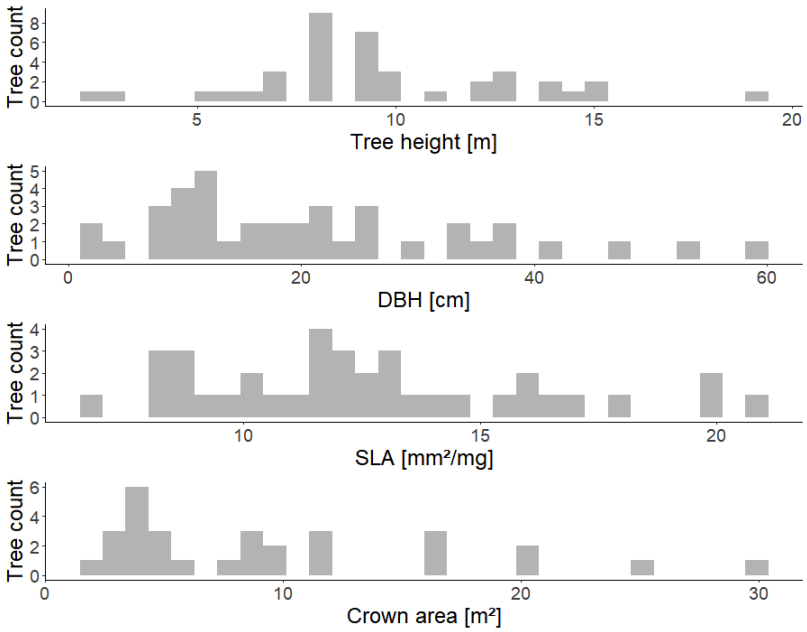


Figure 7.2: Histogram of the tree characteristics from the sampled trees (39) in Northern and Central Peru. The y-axis shows the number of trees and the x-axis gives the range of the variable. Top: Height of the tree (m), top middle: Diameter at Breast Height DBH (cm), bottom middle: Specific Leaf Area (SLA) (mm^2/mg), and bottom: Crown area (m^2).

Each sequence in Table 6.5 was used to search for sequence similarity in GenBank. The BLAST results for only one sample of each population were summarised in Table 7.1 if the obtained BLAST results within one population was equal. The BLAST results of the *rps16* region were not informative, as the best hit of each sample was the same.

Samples from Jaén, Rodríguez de Mendoza, Bongara, and three out of four samples from San Ramón displayed a high similarity with *Cinchona* accessions, in particular with *C. pubescens*. The % similarity with the *C. pubescens* accessions was ≥ 99 % for the majority of the marker regions for samples from the aforementioned geographic regions. The BLAST analysis also revealed that the SAR1 and the TIM samples obtained a high similarity score with accessions belonging to the genus *Ladenbergia* (% similarity = 99 % in 3 out of 4 markers) and no significant similarity was found with the genus *Cinchona*.

The specimens that were retrieved from the plantations in DR Congo displayed no genetic variation in the studied chloroplast regions. The Congolese samples had a high similarity score with *C. calisaya* accessions. However, the ITS region displayed some variability. *Cinchona* sp. 4, 5, and 9, which were grown in the greenhouse in Leuven, obtained a high similarity score with *C. calisaya* accessions (% similarity ≥ 98 for 3 out of 4 marker regions). The other 4 ITS sequences, which were retrieved from plantations in DR Congo, obtained a high similarity score with fungal accessions: *Lasiodiplodia* sp. (% similarity = 100), *Mycosphaerella* sp. (% similarity = 97), and *Fusarium proliferatum* (% similarity = 100).

Table 7.1: Results of the BLAST analyses for each marker. From left to right: sample ID, the number of base pairs of the sequences, the result of the BLAST searches and their accession number, the BLAST scores (the Query Cover (%) and the % Identity), and the voucher numbers with a similar BLAST result.

Code in the paper	#bp	BLAST result	Accession number	Score		Voucher numbers
				Query Cover (%)	% Identity	
matK						
Cinchona sp. SAR1	829	*Ladenbergia* sp.	AY538403	99	99.76	
Cinchona sp. SAR4	763	*Cinchona pubescens*	KY378682	100	99.48	SAR2-3
Cinchona sp. TIM1	805	*Ladenbergia* sp.	AY538403	99	99.88	TIM2-15
Cinchona sp. JAE1	763	*Cinchona pubescens*	KY378682	100	100	JAE2
Cinchona sp. BON3	763	*Cinchona pubescens*	KY378682	100	99.87	BON4;6-11
Cinchona sp. MEN1	763	*Cinchona pubescens*	KY378682	100	99.61	MEN2-8
Cinchona sp. 43	827	*Cinchona calisaya*	AY538379	100	99.88	44, 46, 47, 49, 51, 54, 55, and 57
Cinchona sp. 4	763	*Cinchona calisaya*	KY676289	100	100	5, 9
trnL-F						
Cinchona sp. SAR1	872	*Ladenbergia* sp.	AY538464	99	99.77	
Cinchona sp. SAR4	936	*Cinchona pubescens*	KY378682	100	99.15	SAR2-3
Cinchona sp. TIM1	916	*Ladenbergia carua*	AY538461	100	99.02	TIM2-15
Cinchona sp. JAE1	835	*Cinchona pubescens*	KY378682	100	100	JAE2
Cinchona sp. BON3	939	*Cinchona pubescens*	KY378682	100	100	BON4,6-11
Cinchona sp. MEN1	937	*Cinchona pubescens*	KY378682	100	99.36	MEN2-3,5-8
Cinchona sp. 4	835	*Cinchona calisaya*	KY676264	100	100	5,9
rps16						
Cinchona sp. SAR1	817	*Cinchona pubescens*	KY378682	100	99.66	
Cinchona sp. SAR4	813	*Cinchona pubescens*	KY378682	100	99.75	SAR2-3
Cinchona sp. TIM1	816	*Cinchona pubescens*	KY378682	99	98.41	TIM2-15
Cinchona sp. JAE1	760	*Cinchona pubescens*	KY378682	100	99.61	JAE2
Cinchona sp. BON3	818	*Cinchona pubescens*	KY378682	100	99.63	BON4,6-11
Cinchona sp. MEN1	813	*Cinchona pubescens*	KY378682	100	99.75	MEN2-8
Cinchona sp. 43	821	*Cinchona pubescens*	KY378682	100	99.51	44, 49, 51, 53, 54, 55, 57
Cinchona sp. 4	835	*Cinchona pubescens*	KY378682	100	99.50	5,9
ITS						
Cinchona sp. SAR4	661	*Cinchona pubescens*	MK607898	99	95.75	SAR2-3
Cinchona sp. TIM1	650	*Ladenbergia oblongifolia*	AY538366	92	99.33	TIM2-15
Cinchona sp. JAE1	659	*Cinchona pubescens*	MK607898	100	98.63	JAE2
Cinchona sp. BON3	652	*Cinchona pubescens*	MK607898	100	98.62	BON4,6-11
Cinchona sp. MEN1	649	*Cinchona pitayensis*	AY763888	95	98.08	MEN2-3,5-8
Cinchona sp. 4	594	*Cinchona calisaya*	AY538352	95	98.95	5,9
Cinchona sp. 47	526	*Lasiodiplodia* sp.	MG976369	99	99.81	
Cinchona sp. 49	526	*Lasiodiplodia theobromae*	MK530050	99	100	
Cinchona sp. 51	430	*Mycosphaerella* sp.	KR812238	98	96.71	
Cinchona sp. 55	518	*Fusarium proliferatum*	MN658457	99	99.81	

7.2.2 Nuclear and chloroplast haplotype variation and distribution

Haplotypes were determined for all sequences that demonstrated high similarity (% similarity ≥ 98 %) with *Cinchona* species in the BLAST analyses and for the *C. calisaya* accessions that were retrieved from Maldonado et al. (2017). Seven haplotypes were detected for the chloroplast *rps16* intron (Table 7.2). The nine polymorphic sites in this marker included three base substitutions, four deletions and two repetitive regions. Four haplotypes were found in *Cinchona* spp. originating from its native range in Northern and Central Peru (haplotype A, C, D, and E) and three haplotypes were determined for *C. calisaya* accessions in Bolivia (haplotype B, F, and G). Only one haplotype was detected for *Cinchona* species that originated from the cultivated area within DR Congo. This haplotype was similar to one of the haplotypes that was found in the *C. calisaya* accessions from Bolivia (haplotype B). Within the region of Bongara, two different haplotypes were present: haplotype A and haplotype D. Two base substitutions on position 403 and 455

gave rise to a different haplotype. The specimens of Rodríguez de Mendoza and San Ramón had the same haplotype, haplotype C, which was also the most abundant haplotype in the sampled specimens from Northern and Central Peru. Haplotype E occurred in two specimens that were sampled in Jaén.

Table 7.2: Chloroplast haplotype sequence variation in the *rps16* intron based on a 805 bp alignment. The dash (-) indicates a deletion. Accessions with the corresponding haplotype are listed in the rightmost column.

Haplotype	Polymorphic sites									Voucher numbers
	12	133-135	331	403	455	475	586-594	750	754-762	
A	G	-G-	-	A	G	T	T_9	T	G_8-	BON4 and BON11
B	T	-G-	A	A	G	T	T_7- -	T	G_7- -	*Cinchona* sp. 4, 5, 9, 43, 44, 49, 51, 54, 55, and 57 CMG3998, 4001, 4004, and 4216
C	T	-G-	-	A	G	T	T_8-	T	G_7- -	MEN1-8, and SAR2-4
D	G	-G-	-	G	C	T	T_9	T	G_8-	BON3, and BON6-10
E	G	-G-	-	A	G	T	T_9	-	G_9	JAE1-2
F		AGC	-	A	G	-	T_7- -	T	G_7- -	CMG4214
G		-G-	-	A	G	-	T_7- -	T	G_7- -	CMG4228

The chloroplast *matK* intron comprised five haplotypes (Table 7.3). The 11 polymorphic sites in this marker contained only base substitutions. No indels or repetitive regions were detected. Four haplotypes were found in *Cinchona* spp. originating from its native range in Northern and Central Peru. Only one haplotype was detected for *Cinchona* species that originated from the cultivated area within DR Congo (haplotype B). The same haplotype was found for *C. calisaya* accessions from Bolivia (CMG3998, 4001, 4004, 4214, 4216, and 4228). Within the region of Bongara, two different haplotypes were present: haplotype A and haplotype C. One base substitution on position 111 gave rise to a different haplotype. The specimens from Jaén had the same haplotype as BON4 and BON11 (haplotype A). Three base substitutions on position 193, 432, and 470 gave rise to a new haplotype for the sampled specimens from Rodríguez de Mendoza (haplotype D). An additional substitution on position 482 led to a new haplotype for the sampled species from San Ramón (haplotype E).

Table 7.3: Chloroplast haplotype sequence variation in the *matK* intron based on a 763 bp alignment. The dash (-) indicates a deletion. Accessions with the corresponding haplotype are listed in the rightmost column.

Haplotype	Polymorphic sites											Voucher numbers
	77	104	111	133	193	432	470	482	566	598	637	
A	C	G	C	A	T	C	A	C	G	G	G	BON4, BON11, and JAE1-2
B	T	T	C	C	G	G	A	C	T	T	A	*Cinchona* sp. 4, 5, 9, 43, 44, 46, 47, 49, 51, 54, 55, 57 CMG3998, 4001, 4004, 4214, 4216, and 4228
C	C	G	T	A	T	C	A	C	G	G	G	BON3 and BON6-10
D	C	G	C	A	G	G	C	C	G	G	G	MEN1-8
E	C	G	C	A	G	G	C	T	G	G	G	SAR2-4

The chloroplast *trnL-F* intron covered seven haplotypes (Table 7.4). The 12 polymorphic sites contained 10 base substitutions and 1 indel. No repetitive regions were detected. Five haplotypes were found in *Cinchona* spp. originating from its native range in Northern and Central Peru

(haplotype A, B, C, D, E). Within the regions of Bongara and Jaén, only one haplotype was found. A base substitution on position 165, 262, 623, 648, 825, and 836 gave rise to a new haplotype for the sampled specimens from Rodríguez de Mendoza: haplotype D. Two additional haplotypes were found in MEN6 and MEN8: haplotypes B and C. An additional base substitution on position 117 generated the haplotype E for the sampled species from San Ramón. Only one haplotype was detected for *Cinchona* species that originated from the cultivated area within DR Congo, haplotype F. The same haplotype was found for 4 out of 6 *C. calisaya* accessions from Bolivia. Two base substitutions on position 256 and 667 gave rise to a new haplotype for the two other *C. calisaya* accessions from Bolivia.

Table 7.4: Chloroplast haplotype sequence variation in the *trnL-F* intron based on a 836 bp alignment. The dash (-) indicates a deletion. Accessions with the corresponding haplotype are listed in the rightmost column.

Haplotype	Polymorphic sites												Voucher numbers
	117	165	256	262	427-428	597	623	640	648	667	825	836	
A	C	C	A	A	-C	C	G	A	G	C	T	G	BON3, BON4,BON6-10, BON11, and JAE1-2
B	C	A	A	C	AA	C	A	A	T	C	C	T	MEN8
C	C	A	A	C	-C	A	A	A	T	C	C	T	MEN6
D	C	A	A	C	-C	C	A	A	T	C	C	T	MEN1-3, MEN5, and MEN7
E	G	A	A	C	-C	C	A	A	T	C	C	T	SAR2-4
F	C	A	A	C	-C	C	G	G	T	C	C	T	*Cinchona* sp. 4, 5, 9, CMG3998, 4001, 4004, and 4216
G	C	A	C	C	-C	C	G	G	T	G	C	T	CMG4214 and 4228

The nuclear ITS region comprised 11 haplotypes with 14 polymorphic sites (Table 7.5). The specimens sampled in Bongara were all defined by haplotype A. Only one haplotype was detected for *Cinchona* species that originated from the cultivated area within DR Congo, haplotype B. Two additional substitutions on position 436 and 438 induced a new haplotype for the sampled specimens from Jaén: haplotype C. Three haplotypes were found for the sampled species from Rodríguez de Mendoza: haplotype D, F, and G. An additional haplotype was defined for specimens from San Ramón: haplotype E. Four distinct haplotypes were found for *C. calisaya* accessions: haplotype H, J, K, and L.

Table 7.5: Nuclear haplotype sequence variation in the ITS region based on a 606 bp alignment. The dash (-) indicates a deletion. Accessions with the corresponding haplotype are listed in the rightmost column.

Haplotype	Polymorphic sites														Voucher numbers
	11	26	29	36-50	144	205	248	315	342	437-439	481-489	538	560	569	
A	-	G	T	G-A_3CT-C_4GTC	C	C	G	G	T	TGA	TATGC_2ATG	T	T	T	BON3-4, and BON6-11
B	-	C	-	G-A_3CT-C_4GTC	C	C	G	G	T	TGC	$(\text{-})_3$G$C_2(\text{-})_3$	C	C	T	*Cinchona* sp. 4, 5, and 9
C	-	G	C	G-A_3CT-C_4GTC	C	C	G	G	T	CGA	TATGC_2ATG	T	T	T	JAE1-2
D	-	G	A	–A_3CT-C_4GTC	T	C	G	G	T	TGC	$(\text{-})_3$G$C_2(\text{-})_3$	T	T	C	MEN1-3
E	-	G	A	GTA_2-C$T_2$$C_4$KTY	T	C	G	T	T	TGC	$(\text{-})_3$G$C_2(\text{-})_3$	T	T	T	SAR2-3
F	-	G	A	–A_3CTC_5GTC	T	C	G	G	T	TGC	$(\text{-})_3$G$C_2(\text{-})_3$	T	T	C	MEN5
G	-	G	A	–A_3CT-C_4GT_2	T	C	G	G	T	TGC	$(\text{-})_3$G$C_2(\text{-})_3$	T	T	C	MEN7
H	-	G	-	G-A_3CT-C_4GTC	C	C	G	G	T	TGC	$(\text{-})_3$G$C_2(\text{-})_3$	C			CMG4004 and 4228
J	-	G	-	–A_3CT-C_4GTC	C	T	G	G	C	TGC	$(\text{-})_3$G$C_2(\text{-})_3$	C			CMG3998
K	C	G	-	G-A_3CT-C_4GTC	C	C	T	G	C	TGC	$(\text{-})_3$G$C_2(\text{-})_3$	C			CMG4214
L	-	G	-	G-A_3CT-C_4GTC	C	C	G	G	C	TGC	$(\text{-})_3$G$C_2(\text{-})_3$	C			CMG4216 and 4001

7.2.3 Phylogenetic structure of *Cinchona* samples

The BLAST searches revealed that SAR1 and all TIM samples, which were retrieved from San Ramón and Tingo María, had a high sequence similarity with samples from the genus *Ladenbergia* (% similarity ≥ 99 %) and no significant similarity with samples from the genus *Cinchona*. For this reason, these samples were not taken into account in all downstream analyses.
The combined aligned DNA matrix comprised 3,027 base pairs (bp) derived from the four DNA regions: *matK* (763 bp), *trnL-F* (836 bp), *rps16* (822 bp), and ITS (606 bp). The phylogenetic tree of 21 Peruvian *Cinchona* samples, 12 Congolese *Cinchona* samples, and 14 GenBank accessions (Table 6.5 and 6.6) is shown in Figure 7.3 with posterior probabilities indicated above the branches. Sample *Cinchona* sp. 53 was not included in the topology, as its position within the tree was ambiguous due to missing data.
Three major *Cinchona* groups were observed in the tree with *Ladenbergia oblongifolia* as the outgroup. The first group included all Congolese *Cinchona* samples and the *C. calisaya* accessions which were retrieved from GenBank (PP = 1.0). This group was subdivided into two major clades: clade A containing two *C. calisaya* accessions from GenBank (PP = 1.0) and clade B containing all Congolese samples and four *C. calisaya* accessions from GenBank (PP = 0.979). The second and third group were more closely related to each other than to group 1 (PP = 0.836). The second group was partitioned into 3 major clades: clade C, clade D, and clade E (PP = 0.98). Clade C contained 3 *C. micrantha* accessions that were further partitioned into two subclades (PP = 1.0). Clade D comprised all samples from Rodríguez de Mendoza (PP = 0.883), while the three remaining samples from San Ramón were included in clade E (PP = 1.0). The third group holds 3 clades: clade F, G, and H. Clade F included the 4 *C. pubescens* accessions and the accession of *C. officinalis* that were retrieved from GenBank. However, the *C. officinalis* and *C. pubescens* accessions were not separated into two distinct clades. Clade G comprised 6 out of the 8 samples from Bongara (PP = 0.99). The other two samples collected in Bongara (BON4 and BON11) belonged to the same group (group 3), but were classified into a separate clade (clade F). Clade H covered the two samples from Jaén (PP = 0.8).

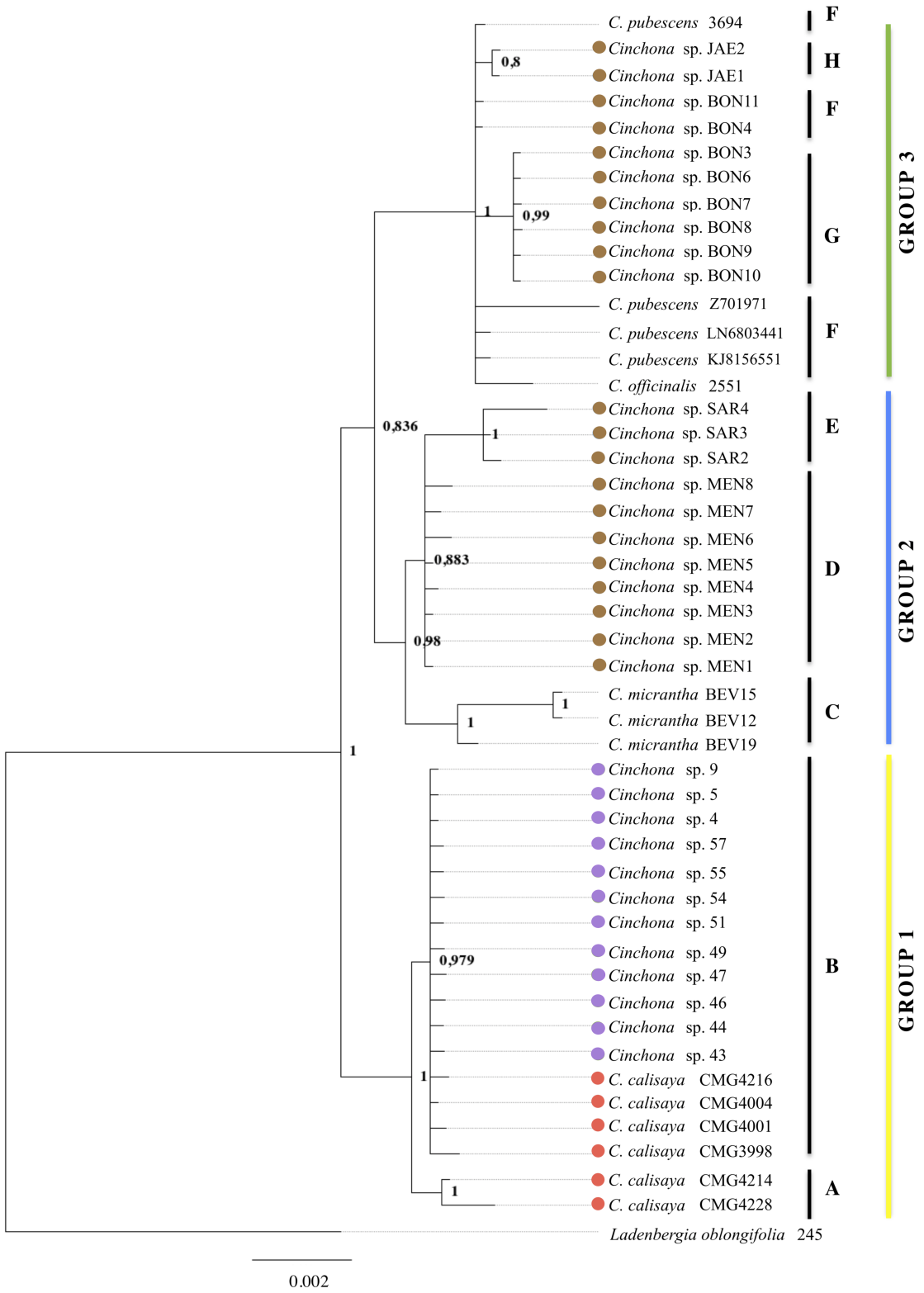


Figure 7.3: Bayesian phylogram (50 % majority-rule consensus) of the *Cinchona* accessions from the sampled regions in Peru (brown circles), the sampled plantations in DR Congo (purple circles), and Genbank based on plastid (*matK, trnL-F*, and *rps16)* and nuclear (ITS) sequences. GenBank accessions from the study of Maldonado et al. (2017) collected in Bolivia are indicated with red circles. *Ladenbergia oblongifolia* 245 was used as outgroup. Node labels indicate posterior probabilities and clade names are shown next to the taxon labels, as well as group names which are indicated in color (group 1: yellow, group 2: blue, and group 3: green).

7.3 Phylogeny linked to environmental traits

Heatmaps of an environmental trait (elevation), a soil property (pH), and three tree characteristics (Specific Leaf Area, mean phosphorous concentration of the leaves, and carbon-to-nitrogen ratio in the leaves) were linked to the phylogenetic tree given in Figure 7.4. The elevation of the sampled *Cinchona* trees varied between 1260 m and 2682 m. The pH of the soil ranged between 2.8 and 5.5. The Specific Leaf Area (mm^2/mg) varied between 5.5 and 21 mm^2/mg. The mean phosphorous concentration of 10 leaves of a sampled tree differed between about 1 and 5 g/kg. The mean carbon-to-nitrogen ratio of 10 leaves of a sampled tree varied between 13 and 43.

Clade A only contained specimens which were collected in Bolivia (red) at an elevation between 1100 en 1392 m. The pH of the soil varied between 4.5 and 5. The second clade, clade B, comprised the remaining specimens which were collected in Bolivia (red), as well as all the collected specimens from the plantations of DR Congo (purple). The Bolivian samples (red) in clade B were collected at higher elevations, ranging from 1700 m to 1900 m. The sampled plantations in DR Congo (clade B, purple) spanned a broader range of elevations, as the elevation varied between 1400 m and 1900 m. The pH at higher elevations in Bolivia was similar to the pH at lower elevations. No pH data was available from the soil of plantations in DR Congo and no data from the tree characteristics was available from the sampled trees from Bolivia in the study of Maldonado et al. (2017). Clade B mainly contained species with SLA values below 10 mm^2/mg with two exceptions in the medium range between 12 and 14 mm^2/mg. The mean phosporous concentration was for the majority of the samples of clade B below 2 g/kg. Three samples in clade B had a mean phosphorous concentration higher than 3 g/kg. The carbon-to-nitrogen ratio varied from the lowest value of 13 to medium values of 30 within the sampled specimens from DR Congo.

The second and third group only contained specimens collected in Peru (brown) and GenBank accessions. The second group is subdivided into three major clades: clade C, clade D and clade E. No data were available for the sequences from GenBank in clade C. The sampled trees from Rodríguez de Mendoza (clade D) were located at an elevation of 1400 m and those from San Ramón (clade E) at higher elevations, around 2000 m. The soil of clade D was very acidic with a pH-H_2O around 3. The pH-H_2O of clade E was more variable ranging from 3 to 5.5. The mean SLA of the sampled specimens from Rodríguez de Mendoza varied in the medium range between 12.5 and 16.5 mm^2/mg, with one exception of 10 mm^2/mg. In San Ramón, the mean SLA values were in the lower range below 10 mm^2/mg. Clade D primarily included trees with a high mean phosphorous concentration, but with a few exceptions around 3 g/kg.

The phosphorous concentration in clade E was very low (between 1 and 2.5 g/kg). Clade D only included trees with a low average carbon-to-nitrogen ratio, whereas the average C/N ratio was highly variable within clade E. The samples which were collected in San Ramón (clade E) included one tree with a low, one with a medium and one with a high average C/N ratio.
Within group 3, three clades were found: clade F, G, and H. Clades F and G comprised the sampled trees in Bongara. All the samples from clade G and clade F were collected at an elevation above 2400m. The pH-H_2O of these soil samples was variable, ranging from 4.4 to 5.5. The average SLA (mm^2/mg) of the samples in clade G and F ranged between 11 and 13 mm^2/mg, with two exceptions. One sample had a mean SLA above 15 mm^2/mg (BON3) and one a mean SLA above 20 mm^2/mg (BON4). The average phosphorous concentration (g/kg) was highly variable for clade G with only very high (> 4.7 g/kg) or very low (< 2 g/kg) concentrations. BON4 of clade F had a medium average phosporous concentration (3.8 g/kg). The carbon-to-nitrogen ratio was low (between 13 and 20) for all samples from clade F and G. Clade H contained the two samples collected in Jaén. These trees were sampled at an elevation of 2300 m and the pH-H_2O of the soil in Jaén was around 4. The average SLA (mm^2/mg) of the leaves was low (between 8 and 10.5 mm^2/mg). The average phosphorous concentration (g/kg) in the leaves was in the medium range, namely around 3 g/kg. The carbon-to-nitrogen ratio was low for this clade (< 20).

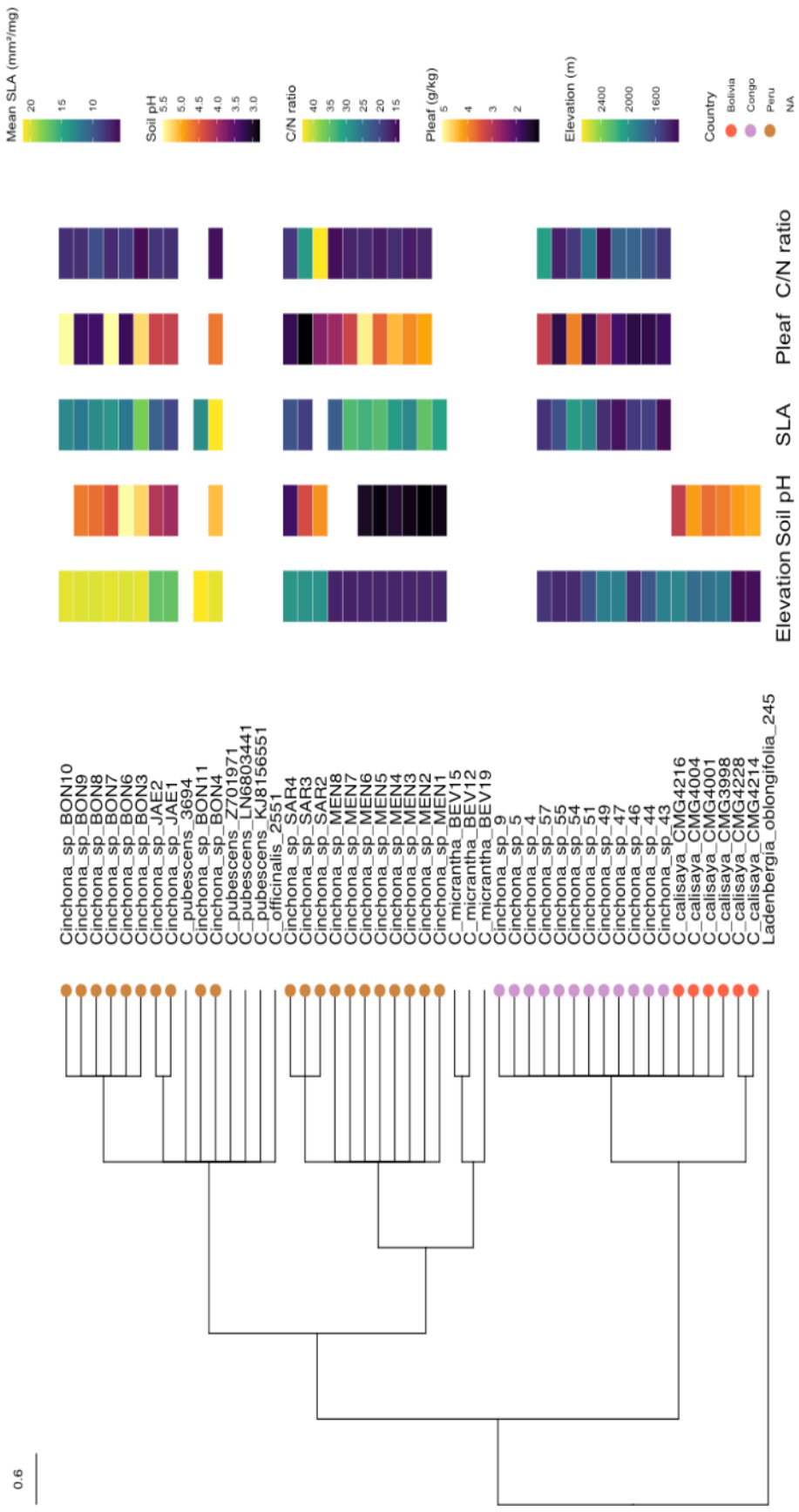


Figure 7.4: Phylogenetic tree linked to environmental traits, tree and soil characteristics. From left to right: Elevation (m), pH of the soil, mean Specific Leaf Area (mm^2/mg), phosphorous concentration of the leaf (g/kg) and the carbon-to-nitrogen ratio in the leaf. The environmental data that were not available is left blank (NA). A subset of the environmental data from Bolivia that were retrieved from Maldonado et al. (2017) was also included. The extraction method prior to pH measurement was not specified in this study (Maldonado et al., 2017). The country where the samples were collected is also indicated with circles: red is Bolivia, purple is DR Congo, brown is Peru, and the unknown (NA) countries are left blank.

7.4 Multivariate analysis and genetic diversity estimation

7.4.1 Principal Component Analysis

The principal component analysis (PCA) of the *Cinchona* accessions and outgroup (Table 6.5 and 6.6) clearly separated the *Cinchona* populations in group 1, 2, and 3 from the outgroup (Figure 7.5). PC1 included 27.31 % of the variability, whereas PC2 contained 25.06 % of the variability. PC1 mainly reflected the separation between *Ladenbergia* spp. and *Cinchona* spp., whereas PC2 predominantly explained the variability between the three groups. The individuals of group 1 were more densely clustered along PC1, PC2, and PC3 compared to the individuals of the other two groups. The third principal component described 15.64 % of the variability. Figure 7.5b and 7.5c show that this principal component (PC3) mainly illustrated the variability within the second and the third group.

The PCA with only the *Cinchona* individuals (without outgroup) is visualised in Figure 7.6. The first principal component (37.48 %) mainly described the variability between the first, the second, and the third group. A clear distinction between the clades within the first and the second group was not visible along PC1 and PC2 (Figure 7.6b). The accessions from clade F in the third group were spread along PC1 and PC2, whereas clade G and H were more clustered together. The second principal component (22.18 %) mainly reflected the variability within the second and the third group, but the clades overlapped with one another within each group. Within group 1, clade A and B were not separated from each other along PC1 and PC2. Figure 7.6c and 7.6d reflect the variability that is described by PC3 (8.24 %). PC3 separated the three clades that could be identified within the second group. One outlier was detected in clade C. Along this first principal component, most samples from clade F did not cluster together with the samples from clade G and H.
The collected samples from Peru were more spread along the first and the second PC, whereas the samples from DR Congo and the *C. calisaya* accessions from Bolivia overlapped (Figure 7.7).

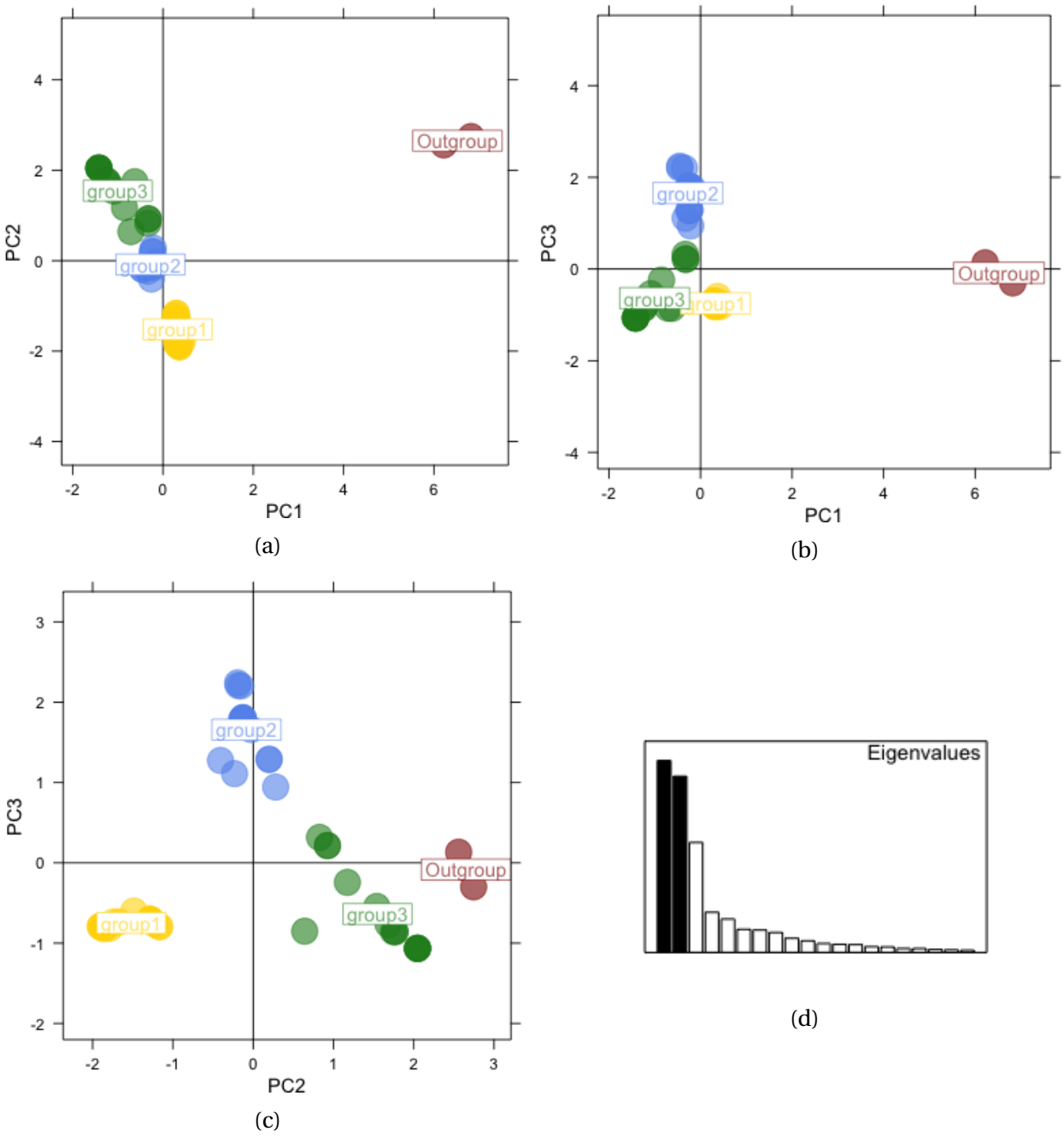


Figure 7.5: PCA plots showing all sampled individuals from Peru and DR Congo and the GenBank sequences, including the outgroup *Ladenbergia*. The groups are all indicated in color according to their clustering in the phylogenetic tree (group1: yellow, group2: blue, group3: green, and outgroup: darkred). (a) PC1 (27.31 %) vs PC2 (25.06 %) (b) PC1 (27.31 %) vs PC3 (15.64 %) (c) PC2 (25.06 %) vs PC3 (15.64 %) (d) Eigenvalues ordered from the first to the last principal component.

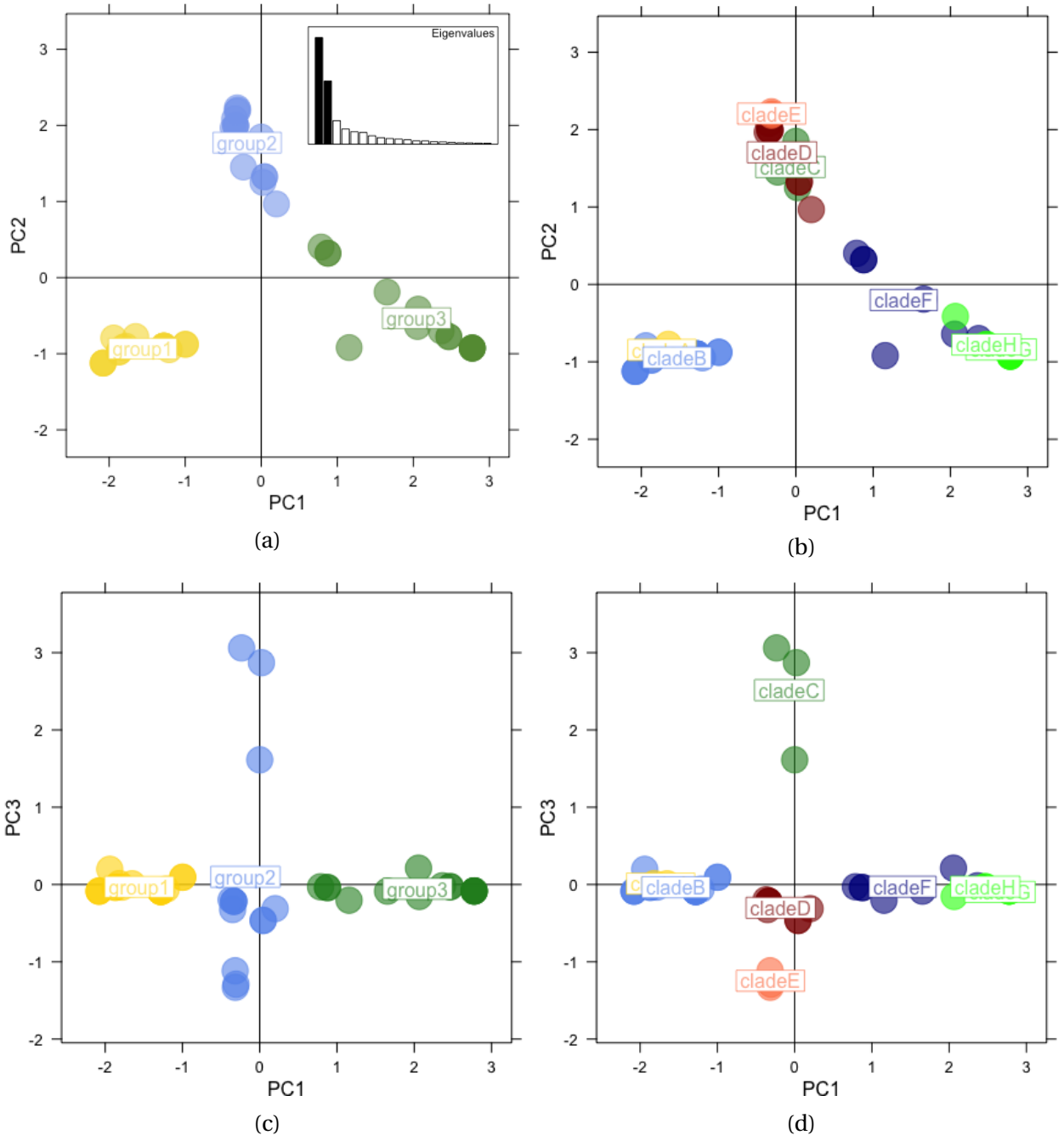


Figure 7.6: PCA plots showing all sampled individuals from Peru and DR Congo and the GenBank sequences, excluding the outgroup *Ladenbergia*. (a) PC1 (37.48 %) vs PC2 (22.18 %) with eigenvalues ordered from the first to the last principal component. (b) PC1 (37.48 %) vs PC2 (22.18 %) (c) and (d) PC1 (37.06 %) vs PC3 (8.24 %). Groups are colored according to their clustering in the phylogenetic tree (group1: yellow, group2: blue, group3: green), clades are also indicated in color (clade A: yellow, clade B: blue, clade C: forestgreen, clade D: darkred, clade E: red, clade F: darkblue, clade G: green, clade H: green).

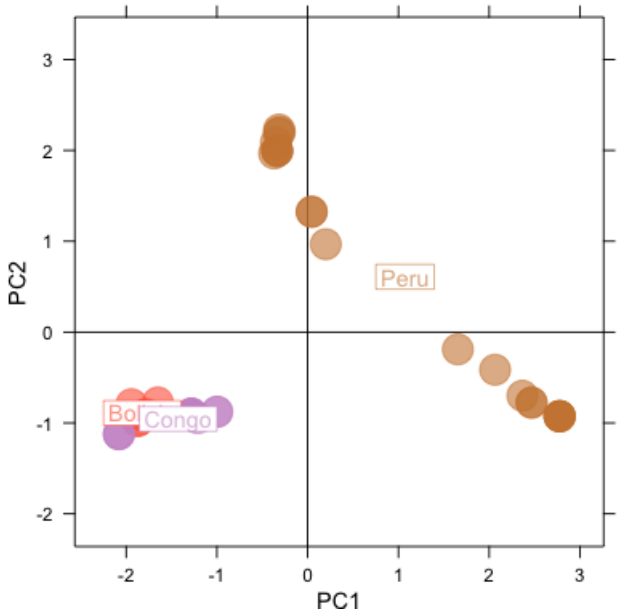


Figure 7.7: PCA plot showing all sampled individuals from Peru (brown) and DR Congo (purple), and *C. calisaya* accessions from Bolivia (red) from the study of Maldonado et al. (2017). PC1 (37.48 %) vs PC2 (22.18 %) with each country indicated in color according to the node labels in the phylogenetic tree.

7.4.2 Allelic Richness

The highest average allelic richness *A* of the sampled populations from Peru was found in the population of Rodríguez de Mendoza (mean $A = 2$), followed by Bongara (mean $A = 1.5$). The average allelic richness for a subset of the sampled populations in Bolivia was 2.5, while the allelic richness in the populations from DR Congo, San Ramón and Jaén was equal to 1.

Table 7.6: Average allelic richness (*A*) of the DNA barcode regions from the studied populations with their standard deviation.

Population	**Mean *A* ± s.d.**
San Ramón	1.0 ± 0
Jaén	1.0 ± 0
Bongara	1.5 ± 0.5
Rodríguez de Mendoza	2.0 ± 1
Bolivia	2.5 ± 1.12
DR Congo	1.0 ± 0

7.4.3 Genetic differentiation estimation: pairwise F_{ST}

Pairwise F_{ST} values were calculated between the delineated *Cinchona* groups. The lowest pairwise F_{ST} was observed between the samples from DR Congo and *Cinchona* accessions from Bolivia clade B ($F_{ST} = 0.52$), whereas the highest pairwise F_{ST} was found between the accessions

from DR Congo and Jaén, DR Congo and San Ramón, and between Jaén and San Ramón (F_{ST} = 1).

Table 7.7: Pairwise F_{ST} values for all pairs of populations of *Cinchona*

	Bolivia clade B	Bongara	DR Congo	Jaén	Rodríguez de Mendoza	San Ramón
Bolivia clade A	0.59	0.74	0.77	0.75	0.75	0.75
Bolivia clade B		0.79	0.52	0.85	0.81	0.85
Bongara			0.84	0.74	0.80	0.82
DR Congo				1	0.89	1
Jaén					0.86	1
Rodríguez de Mendoza						0.82

Chapter 8

Discussion

8.1 Genetic characterisation

8.1.1 Identification of samples in Northern and Central Peru

The BLAST results revealed that the samples collected in San Ramón (SAR1) and Tingo María displayed a high similarity with *Ladenbergia* accessions and a low genetic similarity with *Cinchona* accessions, suggesting that they belong to the genus *Ladenbergia*. These specimens were probably mistakenly identified as *Cinchona* during the field trip. The obtained BLAST results of the samples from Jaén, Bongara, Rodríguez de Mendoza, and three out of four samples from San Ramón suggest that these samples belong to the genus *Cinchona*.
The phylogenetic tree revealed that the collected samples which originated from Central and Northern Peru were included in the second and the third group. The specimens from Jaén and Bongara were clustered together with the *C. pubescens* accessions from GenBank (Figure 7.3), clearly identifying these specimens as *C. pubescens*. Although the number of available sequences of the *C. pubescens* GenBank accessions was limited, the number of informative sites within these sequences was sufficiently high to separate the Jaén and Bongara accessions from most other *Cinchona* species. In the phylogenetic tree, the species *C. pubescens* and *C. officinalis* cannot be distinguished from one another within group 3. The multiple sequence alignment revealed that the variability in the *matK* and *trnL-F* region was nearly absent. However, the ITS region contained multiple base substitutions and deletions in the *C. officinialis* accessions, which were absent in other accessions included in group 3. This is an indication that the *C. officinalis* accession from GenBank was not mistakenly identified, but that there is only a limited amount of variability between *C. officinalis* and *C. pubescens* in the analysed marker regions. Sequence information of the *C. pubescens* accessions was limited, which inhibited the classification of *C. officinalis* and *C. pubescens* into separate clades within the third group.

The sampled population of Bongara and Jaén were separated from each other in the phylogenetic tree. Two exceptions, BON4 and BON11, were clustered apart from the other accessions from Bongara, which showed that intraspecific variation could be detected within one population by using the limited amount of studied marker regions. The principal component analysis revealed the variability within the third group along the first and the second principal component (Figure 7.6). Clade G and clade H were indistinguishable from one another, whereas clade F demonstrated more variability.
Intraspecific variation in *Cinchona* spp. should be further analysed with microsatellite loci, as these regions have been shown to be a powerful tool for the assessment of genetic diversity in tropical trees and the study of population differentiation (White & Powell, 1997; Newton, Allnutt, Gillies, Lowe, & Ennos, 1999). The study of Cueva-Agila et al. (2019) found moderate levels of genetic diversity in *C. officinalis* in the studied populations in the Loja province of southern Ecuador by studying five nuclear microsatellite loci and the chloroplast intron *rps16* (Cueva-Agila et al., 2019). However, specific PCR primers are needed to amplify hypervariable repeat motifs in nuclear or organelle genomes. These microsatellite-containing regions differ between taxa and are currently not available for our species of interest (White & Powell, 1997). Reduced representation sequencing (RRS) methods, such as Genotyping-by-Sequencing (GBS), may also be useful tools to obtain sequence information for a large set of short loci located throughout the genome of *Cinchona* (Dodsworth, 2015). Genome skimming can be used as a complementary tool to RRS, where a limited number of high copy nuclear loci and plastid genomes are completely sequenced (Malé et al., 2014; Dodsworth, 2015). This methodology has the advantage that it can be applied on degraded DNA samples and it has been successfully performed in several cases for taxonomic affiliation (Kane et al., 2012; Besnard et al., 2013; Staats et al., 2013; McPherson et al., 2013; Malé et al., 2014). Complete and accurate characterization of the sequenced samples by using RRS depends on the availability and quality of reference genomes for comparison. Among other things, a reference genome is used to eliminate contamination from the samples by comparing the sequences of the high-throughput data to the reference genome (Yang et al., 2009). However, the major limitation of using HTS on *Cinchona* is that no reference genome sequence of *Cinchona* has been published to date.

The identity of the species that were clustered together within the second group from Central and Northern Peru (three out of four specimens from San Ramón and all specimens from Rodríguez de Mendoza) could not be determined with certainty. However, the structure of the phylogenetic tree suggests that these samples are more closely related to the species *C. micrantha*. The phylogenetic tree indicates that the most recent ancestor of the *C. micrantha* accessions is not the same as the one from all sampled specimens from Rodríguez de Mendoza

and three out of four specimens from San Ramón. This result may indicate that the sampled specimens from Rodríguez de Mendoza and San Ramón belong to another *Cinchona* species instead of being accessions from *C. micrantha*. However, all sampled species from Rodríguez de Mendoza and San Ramón share a recent common ancestor, which suggests that these samples belong to the same *Cinchona* species, but contain some intraspecific variation. All *C. micrantha* accessions share a more recent common ancestor with the sampled specimens from San Ramón and Rodríguez de Mendoza, which indicates that they are more closely related to one another than to the *C. pubescens* and *C. officinalis* accessions from group 3. Group 2 and group 3 also share a more recent common ancestor, which indicates that the *Cinchona* accessions in group 2 are more closely related to the ones included in group 3, in comparison to the accessions in group 1. The phylogenetic structure indicates that the species that are clustered within group 2 are not belonging to *C. calisaya*, neither to *C. pubescens*.
The principal component analysis could also clearly discriminate the three clades from group 2. Clade C comprises one outlier, which is the divergent *C. micrantha* BEV19 accession. Clade D and clade E are densely clustered and can clearly be distinguished from one another along this third PC. However, the two clades are more close to one another in comparison to clade C, which supports the hypothesis that they belong to a different *Cinchona* species than *C. micrantha*. The separation between clade D and clade E is most likely explained by intraspecific variation.

8.1.2 Identity and source population of Congolese samples

The *Cinchona* samples collected in DR Congo were identified as *C. calisaya* based on the BLAST results and their position in the phylogenetic tree. The original *C. ledgeriana* (currently classified as *C. calisaya*) seeds which were donated by the Dutch Queen Juliana, originated from Bolivia (Rocco, 2003). Our results indicate that the *C. calisaya* accessions from DR Congo are not closely related to the Peruvian populations in this study, but that they are similar to the populations in the species' native range in Bolivia. However, no *C. calisaya*'s were sampled in the south of Peru (Figure 1.2). To delineate the region of origin of the cultivated *C. calisaya* more narrowly, a wider sampling of its full native range will be needed in Peru and Bolivia. The Congolese *Cinchona* samples were clustered together with *C. calisaya* specimens in clade B, clearly separated from the *C. calisaya* accessions in clade A. It has to be noticed that the subdivision into clade A and clade B within group 1 is based on only two DNA barcode regions (*matK* and *rps16*) for 9 out of 12 Congolese specimens (*Cinchona* sp. 43, 44, 46, 47, 49, 51, 54, 55, 57). In contrast, all four marker regions (*trnL-F, matK, rps16*, and ITS) were used for the *Cinchona* seedlings (*Cinchona* sp. 4, 5, and 9) and the *Cinchona calisaya* accessions from GenBank (Table 6.5 and 6.6). The availability of these additional two markers further supported the occurrence of the distinct clade A in group 1 (PP = 1.0). Yet, even without the additional information of all marker regions, *C. calisaya*

accessions from DR Congo could be assigned to clade B with a high posterior probability (PP = 0.979). Clade A and clade B were indistinguishable in the principal component analysis and showed only a small amount of variation along the first principal component (Figure 7.6).
The exclusion of sample *Cinchona* sp. 53 suggested that the *rps16* marker contained an insufficient number of informative characters in *Cinchona* to clearly assign samples to their corresponding species. Earlier studies showed that the number of informative characters in the *rps16* marker in other taxa was too low to elucidate phylogenetic relationships between congeneric species (Shaw et al., 2005). In contrast to the *rps16* marker, the *matK* marker seems to have a much higher discriminatory power between species. *Cinchona* sp. 46 and 47 were positioned within clade B with a high posterior probability (PP = 0.979) solely on the sequence information in the *matK* marker.
The ITS accessions from the sampled specimens of DR Congo revealed contamination with fungi. This contamination was primarily with plant pathogens of tropical crops, which may indicate that the contamination was already present in the samples prior to the sample collection. *Lasiodiplodia* spp. are common endophytes and opportunistic pathogens of more than 500 tree species in the tropics and subtropics (Burgess et al., 2006). *F. proliferatum* is known as a pathogen of corn and other crops, and *Mycosphaerella* spp. are associated with leaf diseases of *Eucalyptus* (Park & Keane, 1982; Bacon & Nelson, 1994). Most of these species have not been correlated with disease in *Cinchona* spp. in literature, except for *Fusarium* spp. and *Botryodiplodia theobromae* (syn. *Lasiodiplodia theobromae*) which are registered as secondary pathogens in *Cinchona*'s introduced range in Galápagos (Jäger, 2004).

8.2 Genetic diversity and differentiation

The principal component analysis (PCA) of the full dataset clearly separated the Peruvian (brown) from the Congolese populations (purple) and the Bolivian accessions (red) along the first principal component (PC1) (Figure 7.7). The wider spread of the Peruvian individuals along PC1 and PC2 in comparison to the Bolivian and Congolese samples is explained by several factors. The most important reason is that multiple species of *Cinchona* were sampled in their native range in Peru. In addition, the wider spread of the samples along PC2 in group 2 and group 3 compared to the samples in group 1 suggests a higher genetic variability in *Cinchona* species in their native range (Figure 7.6).

8.2.1 In the native range: Northern and Central Peru

The low allelic richness in the populations from San Ramón and Jaén ($A = 1 \pm 0$) demonstrates the low genetic variability within the sampled populations. The allelic richness was corrected for small sample sizes by using the rarefaction method. However, the sample sizes were too small to adequately correct for this (resp. sample sizes of two and three *Cinchona* trees). These were the only specimens that were found within the region of Jaén and San Ramón, meaning that these samples contain the only genetic diversity present in those regions. As both the number of accessions and the genetic diversity in San Ramón and Jaén were very low, the persistence of *Cinchona* spp. in these regions is regarded as problematic. Although we cannot exclude the possibility that certain trees were not sampled, the number of unobserved *Cinchona* trees in these regions is plausibly very low because of the high search and sampling efforts that were applied during the field work.
The number of sampled species in Rodríguez de Mendoza and Bongara was higher and also a higher allelic richness was determined for the populations from those regions (resp. $A = 2$ and 1.5). These are critical populations for the maintenance of genetic diversity within *Cinchona* specimens. Overexploitation of *Cinchona* in the past for its medicinal use and rainforest clearing are the most important drivers for the loss of genetic diversity within *Cinchona* spp. and other tropical tree species (Acosta Solís, 1945; Tapia-Armijos, Homeier, Espinosa, Leuschner, & de la Cruz, 2015). Although each of the studied populations should be protected from further deforestation and agricultural expansion, the populations within Rodríguez de Mendoza and Bongara deserve the highest conservation priority, because these populations contain the highest amount of genetic diversity. The genetic diversity within *Cinchona* spp. should be maintained within those regions in order to avoid fast genetic erosion. Crop wild relatives are a critical source of genetic variation for improving domesticated plants. Conserving the genetic diversity, and thus the number of alleles, of populations containing crop wild relatives is of utmost importance to maintain genetic variation within species. Consequently, conservation strategies for the protection of these crucial populations are needed (Schoen & Brown, 1993).

The pairwise F_{ST} values can only be interpreted relative to the other values in the matrix (Table 7.7). The pairwise F_{ST} value between Jaén and Bongara ($F_{ST} = 0.74$) indicated a moderate level of genetic differentiation between both populations. A slightly higher level of genetic differentiation was observed for the populations in San Ramón and Rodríguez de Mendoza ($F_{ST} = 0.82$). However, the geographic distance between the populations of Rodríguez de Mendoza and San Ramón is also higher, which might be the reason for a higher genetic differentiation. Genetic diversity can be maintained in fragmented forest populations of *Cinchona* by gene flow via long distance pollination and seed dispersal mechanisms (Kramer, Ison, Ashley, &

Howe, 2008). In addition, isolated trees can function as "stepping stones" between fragmented *Cinchona* populations (Lowe, Boshier, Ward, Bacles, & Navarro, 2005; Lander, Boshier, & Harris, 2010). The winged seeds of *Cinchona* can attain a dispersion distance between 40 - 150 *m* and are probably unable to disperse over longer distances (Vittoz & Engler, 2007). However, rare long distance dispersal events cannot be excluded. Nevertheless, seed dispersal seems to be an unlikely mechanism of gene flow between fragmented *Cinchona* populations in its native range. Pollination of the pink, white, or purple and tubular flowers of *Cinchona* is mainly performed by butterflies and hummingbirds (Sazima, Buzato, & Sazima, 1996; Altshuler, 2003; Tropicos, 2016). Hummingbirds have the capacity to fly over long distances (Murcia, 1996), although they mainly forage within a small area and in unfragmented landscapes (Renner, 1998; Lindberg & Olesen, 2001). However, it remains unknown if large open pastures are impeding hummingbird pollination of fragmented populations. This is presumably also dependent on the hummingbird species (Cueva-Agila et al., 2019).

8.2.2 In the cultivated area: DR Congo

The allellic richness of the cultivated *C. calisaya* accessions from DR Congo ($A = 1 \pm 0$) was smaller in comparison to the allelic richness of *C. calisaya* populations in its native range in Bolivia ($A = 2.5 \pm 1.12$). The determined allelic richness of *C. calisaya* in its native range in Bolivia is presumably an underestimation of the true allelic richness within Bolivia, as this value was determined on a subset of the sampled specimens in Bolivia from the study of Maldonado et al. (2017). Other sequences included in Maldonado's study revealed additional genetic variability. However, we included at least one representative individual from each clade in the phylogenetic tree of Maldonado et al. (2017) in our subset so that most of the genetic variation in the Bolivian *C. calisaya* populations should be present in our study. The allelic richnesses revealed that the genetic diversity within the introduced area is lower in comparison to the genetic diversity in populations within the species' native range in Bolivia. The genetic diversity in DR Congo is limited to one species, *Cinchona calisaya*, and intravarietal genetic variability was absent. Comparable results were yet observed for other crops, such as *Coffea arabica*, where a higher genetic diversity was determined in the wild accessions in Ethiopia in comparison to cultivated accessions (Lashermes, Trouslot, Anthony, Combes, & Charrier, 1996).

Our results demonstrated that no genetic variation was found among *Cinchona* plants collected from different plantations in DR Congo. The clustering of all the Congolese *Cinchona* samples in Clade B and the low allelic richness ($A = 1$) suggest a limited genetic variability of *Cinchona calisaya* in the cultivated area in DR Congo. Tremendous consequences for *Cinchona* cultivation are associated with low crop diversity, as extensively grown monocultures become susceptible to disease and pest epidemics (Altieri, Letourneau, & Risch, 1984). For this reason, regions in

Bolivia (Nor Yungas, Coroico, Chulumani, and Cajuata) possessing wild relatives of domesticated species and containing many varieties of the *C. calisaya* tree should be conservation priorities. If this reservoir of genetic materials is lost, breeding based on natural genetic variation cannot be used to enhance agricultural productivity. In addition, the full native range of *C. calisaya* needs to be explored to characterise its full potential for the improvement of *Cinchona* cultivation in DR Congo (Figure 1.2).

The clustering of the *C. calisaya* accessions of Bolivia with the samples of DR Congo in the principal component analysis confirmed the high genetic similarity between wild and cultivated accessions (Figure 7.7). This was also reflected by the low pairwise Fst value (0.52) indicating low levels of genetic differentiation between the wild populations in Bolivia and the cultivated accessions in DR Congo. This is probably because of the limited amount of breeding efforts that have been performed on the cultivated accessions. However, multiple markers (e.g. microsatellites or SNPs) should be analysed to draw further conclusions about the genetic variation and differentiation between *Cinchona calisaya* in its native and introduced range. The markers used in this study are sufficient to identify the accessions on species-level. However, they do not provide enough information to detect patterns of intraspecific variation.

8.3 Environmental diversity in *Cinchona*

Cinchona plants will establish and regenerate in environments to which they are well adapted. For this reason, their geographic distribution and the distributions of associated functional traits (i.e. morphological, biochemical, physiological, structural, phenological, or behavioural characteristics of organisms that influence performance or fitness), will indicate the distribution of optimal or near-optimal environmental conditions for the growth of *Cinchona* accessions in space and time (Nock, Vogt, & Beisner, 2001; Ackerly, 2003).

The phylogenetic tree revealed clustering in functional and environmental traits (Figure 7.4). Closely related species may have more similar habitat preferences than distantly related species and habitat preferences may also be species-specific (Pei et al., 2011). Within a species, variation is found between populations in either the elevation (m), pH of the soil, SLA (mm^2/mg), phosphorous concentration of the leaf (g/kg), and/or the carbon-to-nitrogen ratio. Competition or opportunity by colonizing a new environment provide niche differentiation between closely related species (Ackerly, 2003).

8.3.1 In the native range: Northern and Central Peru

Cinchona spp. may occur at an elevation range between 800 - 3300 *m* a.s.l. (Jäger, 2004). At higher elevations, the growth form may be stunted. The sampled regions in Peru were located on the eastern slope of the Andean mountain range at an elevation between 700 and 2682 *m*. Different climates are associated with these different elevations (Figure B.1). At higher elevations (up to 2600 *m*), a temperate climate with no dry season and a warm summer is found, whereas at lower elevations (700 *m*) a tropical rainforest climate is encountered. Trees were also found at 1369 *m* in a tropical savannah climate (Beck et al., 2018). Similar climatic conditions were described in literature (Hodge, 1948; Nair, 2010). The temperature of the sampled regions was on average 19.54 °C (s.e. = 0.61 °C) and never dropped below 0 °C as *Cinchona* spp. cannot survive freezing temperatures (Acosta Solís, 1945; Nair, 2010). In addition, the sampled regions are characterized by a high amount of precipitation (1501, s.e. = 103 $mm/year$), which is characteristic for the eastern slope of the Andean mountains (Figure 7.1 and B.2) (Emck, 2007; Garreaud, 2009). The surrounding environment of the sampled trees in *Cinchona*'s native range was highly variable. Some trees were located in dense forests, whereas others occurred as isolated individuals in pastures. Interviews with local people (Appendix A) suggested none of the sampled trees were planted. Several trees were located in an environment that was altered for cultivation.
The pH-H_2O of the soil in the sampled regions in Peru was on average 4.01 (s.e. = 0.15). Highly acidic soils are typical for tropical regions. However, the soil of the sampled *Cinchona* populations was on average more acidic, especially in Rodríguez de Mendoza (average pH-H_2O = 2.91, s.e. = 0.033), in comparison to previous studies, which reported an average pH of 5.1 (Acosta Solís, 1945; Nair, 2010). The acidic environment in Rodríguez de Mendoza is attributed to the accompanied Tocuya hot sulfur springs (Fliermans & Brock, 1972). The pH of the soil of the *C. calisaya* accessions from Bolivia (clade A and B) was in a similar range as the pH-H_2O of the Peruvian soil samples of clade E, G, and H. However, the extraction method prior to pH measurement was not specified in the study of Maldonado et al. (2017). The used solution (either KCl, $CaCl_2$, or H_2O) may strongly affect the measured pH value, as it can result in a difference of one unit (Gavriloaiei, 2012).

The SLA is an appropriate leaf trait that is correlated with several leaf attributes, and is the most dominant factor that explains variation in relative growth rate (Beadle, 1993; Bhadouria, Tripathi, Srivastava, & Singh, 2019). Previous studies have demonstrated that the growth of *Cinchona* trees is highly affected by the ambient light condition and the climate (Nandi & Chatterjee, 1991). The highest SLA values were found for those *Cinchona* species that were sampled at lower elevations in Rodríguez de Mendoza (1400 *m*), with two exceptions (BON3 and BON4), which were sampled at higher elevations above 2400 *m* in Bongara (Figure 7.4). The SLA was lower

for the remaining specimens from Bongara, Jaén, and San Ramon, which were all located at higher elevations. This result suggests that lower elevations favor the relative growth rate of *Cinchona* trees. The study of Maïa De Ridder confirmed these findings and revealed that the SLA was higher at higher temperature, higher precipitation, and lower elevation (De Ridder, 2020). However, temperature was highly correlated with precipitation and elevation. Previous studies showed similar trends when the effect of elevation on SLA was analysed (van de Weg, Meir, Grace, & Atkin, 2009; Asner & Martin, 2016; Bauters et al., 2017). In addition, the SLA, and thus the growth of the *Cinchona* tree, was also highly affected by the ambient light condition. The results of Maïa De Ridder suggest that shade would presumably increase the SLA and thus growth of the tree. This result is in concordance with previous studies in *Cinchona* plantations, where the relative growth of the tree increased by adding more shaded trees (Nandi & Chatterjee, 1991). However, we should bear in mind that we are comparing different *Cinchona* species which might also affect the SLA value.

The canopy cover also affects the nutrient composition of the leaves. The phosphorous concentration of the leaves varied within a population in the phylogenetic tree (Figure 7.4). A higher mean leaf phosphorous concentration was found for trees with a denser canopy (De Ridder, 2020). A previous study on *Theobroma cacao* in Ghana demonstrated that shade increased the levels of nitrogen, phosphorous, and potassium in the leaves (Burridge, Lockard, & ACQUAYE, 1964). The C/N ratio in the leaves was more or less stable for all groups in the phylogenetic tree. This ratio is predominantly determined on genus level. A significant lower carbon-to-nitrogen ratio was found in the leaves of the *Cinchona* specimens in comparison to the leaves of *Ladenbergia* specimens in the study of Maïa De Ridder. This study also revealed that the N/P ratio also remained stable for all *Cinchona* clades, whereas a significant higher value was found for the *Ladenbergia* specimens. The leaf N/P ratio has widely been used as indicators of limitations in the relative availability of environmental N and P (Sullivan et al., 2014). However, the study of Yan, Tian, Han, Tang, and Fang (2017) demonstrated that the relation between the N/P ratio and nutrient availability is not always reliable.

8.3.2 In the cultivated area: DR Congo

The elevation of the 125 plantations in DR Congo varied between 1412 and 1904 m and was on average 1619.13 m (s.e. = 15.59 m). The cultivated area of *Cinchona* trees in DR Congo was predefined by humans. Factors that determine the location of the cultivated area are either favorable environmental conditions for *Cinchona* cultivation, but also human demographic distribution, accessibility, and political situations. Within this elevational range, the mean annual temperature was 23.04 °C (s.e. = 0.17 °C) and the annual precipitation pattern is more or less constant (1705.12, s.e. = 16.16 $mm/year$), but the frequency of rainfall throughout the year

varies because of the different climates that are found within this elevational range (Figure B.4). Some plantations are found in a temperate climate with a warm summer and either a dry winter or a dry summer. Others are located in a tropical savannah climate (Figure B.3) (Beck et al., 2018). Further studies need to sample and analyze soil data of plantations of DR Congo in order to compare this data with its native range.
The mean SLA (mm^2/mg) within DR Congo did not vary a lot along the elevation gradient. The mean SLA was lower for *Cinchona* specimens sampled in the plantations in DR Congo than in those sampled in Peru. However, we are comparing different species of the genus *Cinchona*, which might also be an important determining factor for observed SLA differences. No SLA data was available for *C. calisaya* specimens sampled in its native range. Previous studies have observed that the SLA in the cultivated area is often lower in comparison to the natural habitat (Kufa & Burkhardt, 2011; Gagliardi, Rapidel, Virginio Filho, & Isaac, 2014; Martin & Gandara, 1945). A study on *Coffea arabica*, a species that is also part of the Rubiaceae family, demonstrated that the SLA of trees in their natural habitat in Ethiopia was higher in comparison to the SLA of trees in their introduced range in plantations in Costa Rica and Nicaragua. However, when the shade increased in the plantations, the SLA increased as well, which suggests that shade is an important determining factor for the SLA and thus the relative growth rate of the plant (Kufa & Burkhardt, 2011; Gagliardi et al., 2014; Martin & Gandara, 1945).
The leaves of the trees in DR Congo were also characterized by a similar C/N ratio as the trees in Peru, which supports the result that the C/N ratio is presumingly determined on the genus level. No data was available for the nutrient composition in the leaves of *C. calisaya* accessions from the study of Maldonado et al. (2017).

The study of Maïa De Ridder demonstrated a significant correlation between the phylogenetic clustering of accessions in clades and their variation in SLA and leaf carbon content. However, the leaf carbon-to-nitrogen ratio did not differ between the clades. This may indicate that trees compensate for the increased uptake of leaf carbon by taking up more leaf nitrogen as well. This study extensively analysed the effect of environmental factors on the functional traits of *Cinchona* trees in their natural environment in Northern and Central Peru and its introduced area in DR Congo (De Ridder, 2020).

8.4 Effect of environment and phylogeny on the alkaloid concentration in the *Cinchona* bark

The subdivision of group 1 into two clades was also demonstrated in the study of Maldonado et al. (2017). This study revealed that clade A includes *C. calisaya* trees with a high alkaloid content

(13.6-41.3 $mg\ g^{-1}$ cumulative weight of four major alkaloids) and clade B primarily includes trees with a lower alkaloid content (0.3-12.5 $mg\ g^{-1}$ cumulative weight of four major alkaloids), although there were a few exceptions (Maldonado et al., 2017). These results give an idea of the plausible alkaloid concentration of the sampled trees in the plantations of South Kivu. The genotype of clade A is the high-yielding genotype from Bolivia and might be better for cultivation purposes in DR Congo, as higher alkaloid concentrations were observed for the corresponding genotype in *C. calisaya*'s native range. However, the low-yielding variety from clade B should not be discarded by the farmer, as a strategy of risk management in uncertain farming conditions.
Apart from the genotype, environmental factors might also have an influence on the alkaloid concentration found in the bark. Clustering was observed in several environmental traits in the phylogenetic tree (Figure 7.4). Studies that analyse the effect of the environment on the alkaloid content have been carried out on plantations to optimise alkaloid production. For instance, the elevation of plantation sites might affect the alkaloid concentration, as demonstrated by Yonzone and Chatterjee (1985) in Indian plantations. The bark yield of *C. ledgeriana* (syn. *C. calisaya*) increased at higher elevations. In addition, the study of Chatterjee, Nandi, Choudhuri, and Chatterjee (1979) demonstrated that *Cinchona* trees grown at higher elevations contained higher alkaloid concentrations when they were planted on north and east facing slopes, whereas *Cinchona* trees grown at lower elevations were higher yielding when planted on south and west facing slopes. The results of a more recent study of Maldonado et al. (2017) were in contrast to those of Yonzone and Chatterjee (1985). A high-yielding variety of *C. calisaya* was sampled at an elevation between 1100 and 1392 m, whereas a low-yielding variety was sampled at higher elevations between 1700 m - 1900 m. The elevational range of the sampled plantations in DR Congo varied between 1400 and 1900 m. Maldonado et al. (2017) described both the effect of environment and genotype as well. However, they concluded that the genotype of the species is the major driver of differences in alkaloid content between congeneric species in its native range in Bolivia (Maldonado et al., 2017). Furthermore, a higher nitrogen level in the soil led to higher amounts of total alkaloid and quinine sulfate in *Cinchona* trees, as shown by Loustalot and Winters (1948). However, no soil data were collected in the sampled plantations of DR Congo. The study of Maïa De Ridder focused on the analysis of environmental factors that have an influence on the growth of *Cinchona* trees in their native range in Northern and Central Peru. This study can also serve as a basis to assess the environmental factors that might affect the alkaloid concentration in *Cinchona* trees.
After all, the possible occurrence of pathogens in the *Cinchona* populations in DR Congo might have a positive or a negative effect on the alkaloid concentration in the bark, as alkaloids are thought to play an important role in the defense against herbivores and pathogens (Zulak et al., 2006). The health of the tree also plays an important role, especially during the early stages of

development, and pest control during these early stages is of major importance (Chopra & Peter, 2005). The suboptimal conditions of the quinine production in the plantations in DR Congo might have other reasons apart from the genotype. Moreover, the optimal genotype for cultivation might also differ from region to region. For the aforementioned reasons, environmental factors have to be considered as well when bark samples are analysed for their alkaloid content with HPLC.

Chapter 9

Conclusion and future perspectives

From the laboratory and data analysis, we concluded that appropriate storage of the leaf samples prior to the DNA analysis is of major importance to successfully extract DNA and perform amplification of the predefined marker regions.
We identified the populations of Jaén and Bongara in Peru on species level as *Cinchona pubescens*, whereas the Peruvian populations from San Ramón and Rodríguez de Mendoza belong to the genus *Cinchona* but were not identified as *C. pubescens*. The phylogenetic tree illustrated the phylogenetic patterns in *Cinchona* across populations in Northern and Central Peru, as well as in DR Congo. However, genetic variability within the studied populations was limited. The amount and distribution of genetic variation within a species are of critical importance to its evolutionary potential, and determine its chances of survival (Holsinger & Gottlieb, 1991). Efforts to educate local population and landowners about the importance of conserving the few remaining populations of this threatened genus should continue because biodiversity and human health are linked to one another. Further research need to be performed by using High Throughput Sequencing techniques and analyzing microsatellite regions to reveal population structure on a finer genetic scale. The studied marker regions enabled us to identify sampled specimens on species level. Nevertheless, they did not provide enough information to detect patterns of intraspecific variation.
In addition, more populations and more individuals per populations, if available, must be analyzed to obtain a more complete view on the genetic diversity and differentation in *Cinchona* spp. in their native range. Our results demonstrated that the highest genetic diversity was found in Rodríguez de Mendoza and Bongara, located at an elevation of resp. 1400 m and above 2400 m. Both regions were associated with an acidic soil (mean pH = 4.02, s.e. = 0.15), a high temperature (19.54, s.e. = 0.61 °C) and a high amount of precipitation (1501, s.e. = 103 $mm/year$). These environmental conditions favour the regeneration of *Cinchona* specimens, as was demonstrated by previous studies (Acosta Solís, 1945; Jäger, 2004). Bongara and Rodríguez de Mendoza are the

two regions that deserve highest conservation priority because they contain the highest number of alleles. However, each studied population need to be protected from further deforestation and agricultural expension. Remaining fragments of *C. pubescens* and *C. calisaya* need to be sampled to correctly estimate the complete genetic diversity in their native range in Ecuador, Peru, and Bolivia (Figure 1.2). This will help us to better understand the conservation needs of the genus *Cinchona*.

The sampled trees from plantations in DR Congo were identified as *C. calisaya* and a low intraspecific genetic diversity was estimated between the trees in those plantations. On top of that, the cultivated genotype equals the low-yielding variety of *C. calisaya* in its native range in Bolivia. Cultivating the higher yielding *C. calisaya* variety from clade A might increase the quinine yield. Nevertheless, we might not neglect that environmental factors might also affect the alkaloid production. In addition, the cultivated genotype from South Kivu might be an optimized variety for cultivation with higher alkaloid yields. The measurement of the alkaloid concentration in the bark samples needs to be performed to shed some light on these results. Furthermore, low-yielding varieties should not be discarded by the farmer for the sake of genetic diversity, as it can be integrated into risk management strategy to cope with uncertain farming conditions (e.g. changing environmental circumstances). A wider sampling of *C. calisaya*, preferably from its full native range in Bolivia and the south of Peru, will be needed to identify new genetic variation for the enrichment of the cultivated *Cinchona* gene pool (Figure 1.2).

The COVID-19 crisis impeded the laboratory work that had to be performed on the *Cinchona* bark samples in order to estimate the alkaloid concentration, and to investigate whether a phylogenetic signal is present in the alkaloid production. Studies that are analyzing both environmental and genetic factors in function of optimal alkaloid production in *Cinchona* spp. are lacking. This phylogenetic study can be used as a strong basis for the assessment of phylogenetic patterns of alkaloid production in *Cinchona*.

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Appendices

A Local experts in Northern and Central Peru

Sampled areas in Northern and Central Peru were chosen by Daniel Angel Armas, who interviewed local experts of *Cinchona* spp. :

- Joaquina Alban, PhD – Head of the Department of Ethnobotanic and Professor at the Museum of Natural History at the Universidad Nacional Mayor de San Marcos (UNMSM), Lima (currently working actively on *Cinchona* L. genus);
- Carlos Reynel, PhD – Dendrologist, Etnobotanist and Professor in Forest Sciences Faculty at UNALM, Lima;
- Aniceto Daza – Technical support at the MOL (UNALM) Herbarium, Lima; Dennis del Castillo Torres, PhD – senior researcher at the Research Institute for the Peruvian Amazon / Instituto de Investigaciones de la Amazonía Peruana (IIAP);
- John Remuzgo, Agr. – junior researcher at the Research Institute for the Peruvian Amazon / Instituto de Investigaciones de la Amazonía Peruana (IIAP); Eduardo Chica, PhD. – Horticultural Science specialist, researcher and Professor at Cuenca University

B Maps

B.1 Northern and Central Peru

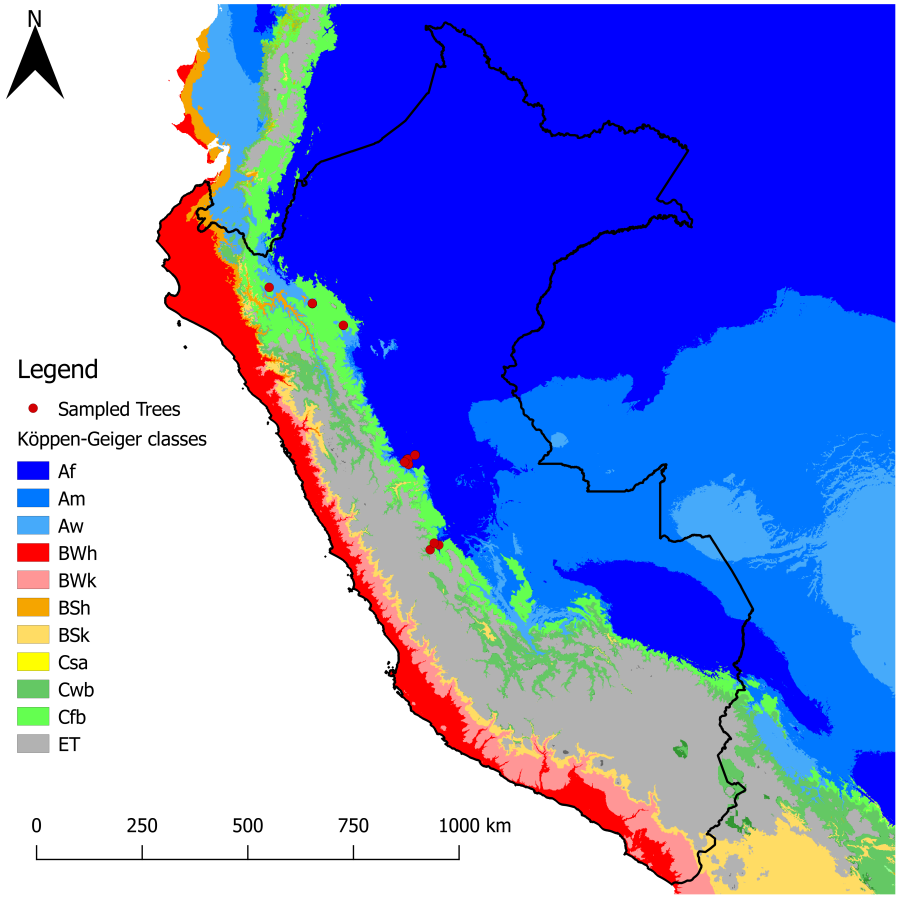

Figure B.1: Map of Peru with the Köppen-Geiger climate classification at an 1-km resolution for the present-day (1980–2016) by Beck et al. (2018). The sampled *Cinchona* trees are indicated on the map (red dots). Only the visible climate classes are given in the legend. The trees of Tingo María and San Ramón were located in a region with a tropical rainforest climate (Af), those of Jaén and Bongara were in a temperate climate with no dry season and a warm summer (Cfb) and finally those in Rodríguez de Mendoza were in a tropical savannah climate (Aw).

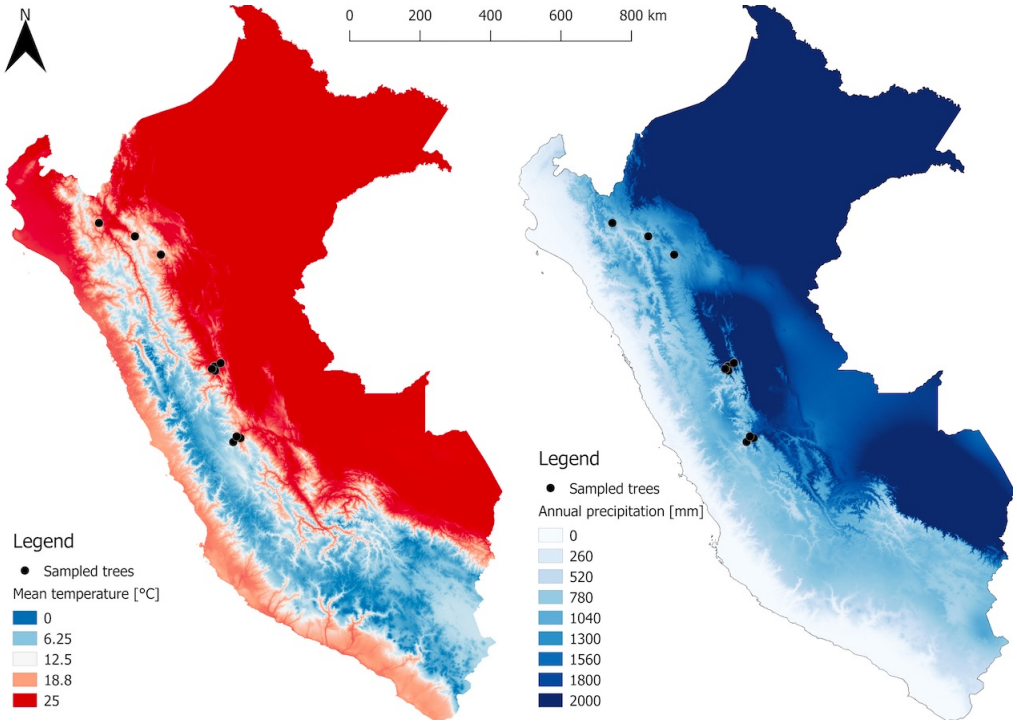

Figure B.2: Maps of Peru showing the mean annual temperature (°C) (left) and the annual precipitation (mm) (right) based on 30 s resolution data for the period 1960-2018 from WorldClim. Sampled trees (39) are indicated with black dots.

B.2 DR Congo

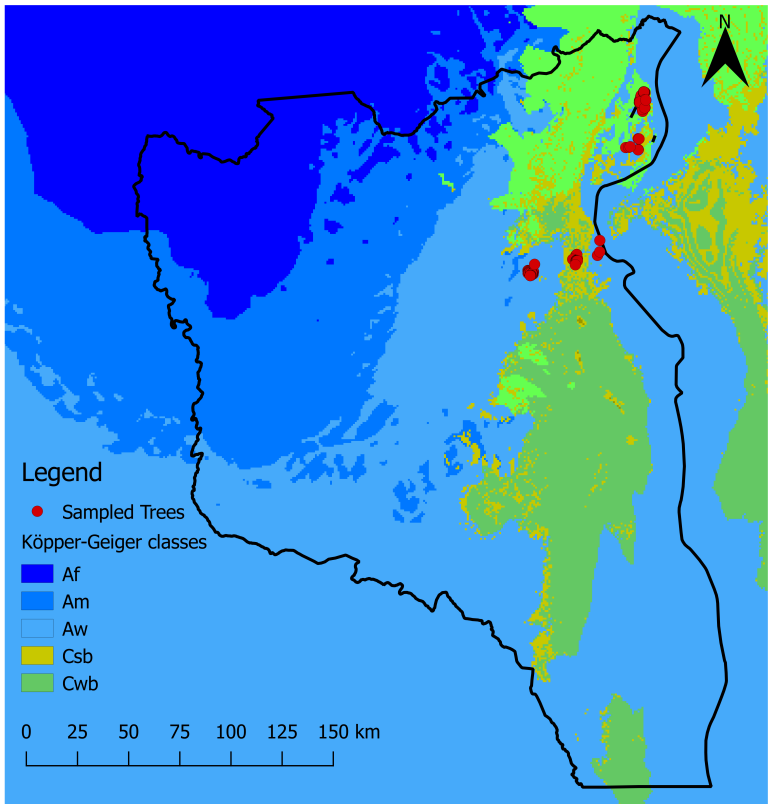

Figure B.3: Map of South Kivu, DR Congo with the Köppen-Geiger climate classification at a 1-km resolution for the present-day (1980–2016) by Beck et al. (2018).The sampled *Cinchona* trees are indicated on the map (red dots). Only the visible climate classes are given in the legend. Sampled trees were located in either tropical savannah (Aw), a temperate climate with a dry winter and a warm summer (Cwb) or a temperate climate with a dry and warm summer (Csb).

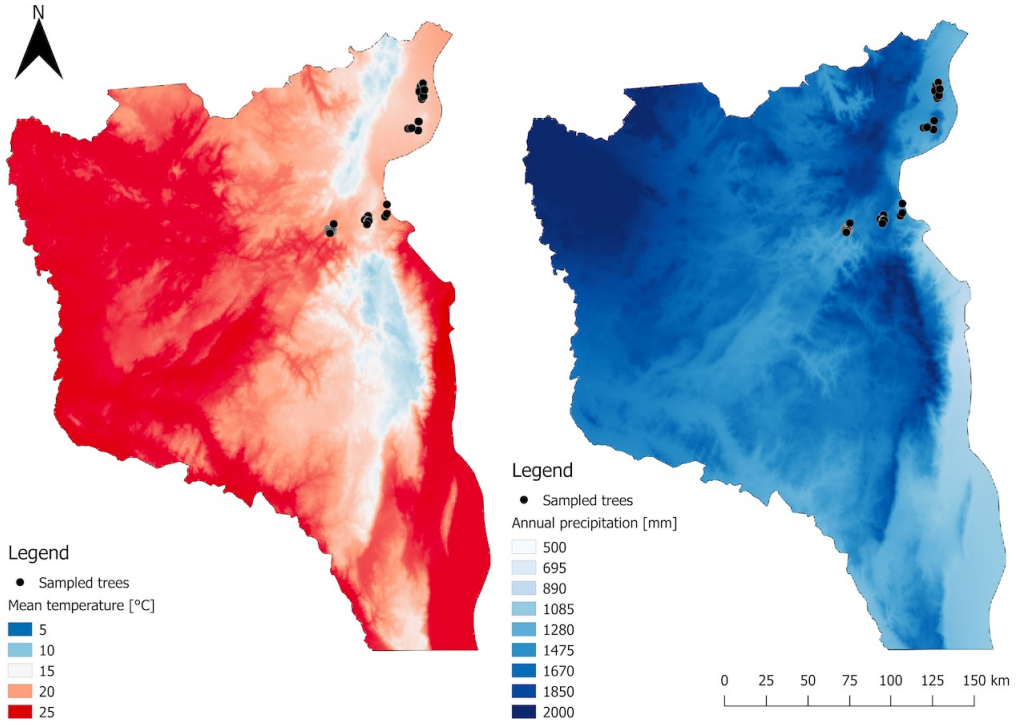

Figure B.4: Maps of South Kivu, DR Congo showing the mean annual temperature (°C) (left) and the annual precipitation (mm) (right) based on 30 s resolution data for the period 1960-2018 from WorldClim. Sampled trees (125) are indicated with black dots.