Master's thesis

NTNU
Norwegian University of Science and Technology
Faculty of Natural Sciences
Department of Biology

Marthe Svihus

DNA barcoding of weevils (*Coleoptera: Curculionidae*) to disentangle the pollination network of the weevil-aroid community in a lower montane rainforest in Colombia

Master's thesis in Natural science with teacher education
Supervisor: Glenn Dunshea
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Preface

UN's Sustainable Development Goal 15 is conserving life on land, implying "sustainably manage forests" and "stop biodiversity loss". This Sustainable Development Goal, emphasizes a climate change and biodiversity loss crisis (United Nations, n.d.-b). Additionally, there are several knowledge gaps within weevils in Colombia, and its pollination, especially on a genetic level. This study contributes to increasing the understanding of biodiversity in one of the countries with the highest biodiversity in the world (Noreña – P et al., 2018). The study also facilitates further research based on the molecular data on weevils. This study will also independently contribute to better knowledge about weevils' genetic diversity, by later publishing DNA barcodes in the BOLD system. A better understanding of weevil biology and systematics will contribute to the understanding of the lower montane rainforest ecosystem in Colombia, further contributing to better conservation methods for this ecosystem and its biodiversity.

Furthermore, UN's Sustainable Development Goal 4 is quality education, which is crucial for being able to achieve the other Sustainable Development Goals as the life on land goal described above (United Nations, n.d.-a). Experiences from lab work from this study can contribute to better practical science education in school as a teacher. Continuously, a part of the curriculum in the Norwegian education system concerns respect for nature and environmental awareness (*Ministry of Education and Research, 2017, p. 8*). This study can be utilized in Norwegian education to give students more insight into the complexity and diversity of nature, contributing to students' respect for nature. This study can additionally show students the importance of cooperation between countries to be able to solve global problems such as biodiversity loss, which is reflected in the UN Sustainable Development Goal 17.

Acknowledgments

This project started in August 2023, with a field trip to Cali, Colombia. First, I would like to thank my co-supervisor Alejandro Zuluaga Trochez for being so welcoming, introducing me to Colombian culture, and helping me with my thesis. I am so grateful for getting the opportunity to travel to Colombia with my master, and this travel will be an experience I will not forget. A big thanks to Michael for giving me this BigTREE project and thesis as an opportunity, and for helping me plan the trip to Colombia.

Continuously, I want to give a big thanks to my supervisor Glenn Dunshea for coming to Colombia to help me during fieldwork and for helping me with lab work and data analysis. I would also like to thank Kamal, Allison, Camilo, Alejandro, and Glenn for helping me with fieldwork in Colombia. I also want to thank Torbjørn Ekrem, Mohsen Falahati, and Ottavia for helping me with lab work, and Frode Ødegaard for helping me with Weevil's systematics. I also want to give thanks to the people at Universidad del Valle and NTNU Science Museum, Department of Natural History for being so welcoming, during my stay in Cali and my lab work in Trondheim.

It has been 5 memorable years at LUR, ending with a master project that I found highly interesting. I want to thank my fellow students and friends Lina, Andrea, Julie, Mari, and Aurora. These 5 years at NTNU wouldn't be the same without you. Finally, I also want to thank Øystein for continuously supporting me during the work with this master thesis.

Abstract

The Colombian lower montane rainforest includes a highly specialized and complex weevil-*Araceae* pollinating system with brood site pollination mutualism (BSPM). The weevil tribes *Derelomini* and *Acalyptini* (*Coleoptera: Curculionidae*), have been described morphologically as flower-pollinating weevils with several observed interactions with *Araceae* inflorescence. However, it has been difficult and time-consuming to characterize the *Araceae*-pollinating weevils, based on morphospecies and genitalia, as there is a knowledge gap concerning these weevil species, their genetic diversity and specialized pollination interactions. This project presents DNA barcoding by PCR and DNA sequencing of the COI mitochondrial gene on 41 weevil specimens collected from 10 different *Araceae* species' inflorescence in a lower montane rainforest in Colombia. Phylogenetic analysis split the weevil specimens into 10 highly differentiated haplotype groups based on their genetic distance. The phylogenetic analysis results show clear, separated haplotype groups of specimens confirmed as being separate species, supported by morphological data. Additionally, one haplotype group is identified to *Cyclanthura cf. oculata*. Some haplotype groups demonstrate cryptic diversity, where previous identifications into morphospecies were shown to be inaccurate. The *Cyclanthura* species' pollination pattern could indicate a specialized pollination interaction with *Araceae*, where each haplotype group was found pollinating on 1-2 *Araceae* plant species, although survey and sample sizes were too small to confirm specialized interactions. This observation is hypothesized to be partly driven by potential reproductive barriers and adaptation through evolution.

Sammendrag

I lavere fjell-regnskoger i Colombia eksisterer det høyt spesialiserte og komplekse snutebille-*Araceae*-pollineringssystem med avkoms-sted pollinerings mutualisme (BSPM). Snutebille-stammene *Derelomini* og *Acalyptini* (*Coleoptera: Curculionidae*), er beskrevet morfologisk som blomster-pollinerende snutebiller med flere observerte interaksjoner med *Araceae* blomsterstand. Det har vist seg å være vanskelig og tidkrevende å karakterisere *Araceae*-pollinerende snutebiller basert på morfologiske art og kjønnsorganer. Det er i tillegg er mangel på kunnskap om disse snutebille-artene, deres genetiske diversitet og deres spesialiserte pollineringsinteraksjoner. Denne studien presenterer DNA barkoder, basert på PCR og DNA sekvensering av COI mitokondrie-gen fra 41 snutebille eksemplarer innsamlet fra 10 ulike *Araceae* plantearter fra en lavere montane regnskog i Colombia. Fylogenetisk analyse splittet snutebille-eksemplarene til 10 høyt differensierte haplogrupper basert på deres genetiske avstand. Resultatene fra den fylogenetiske analysen som viser tydelige, separerte haplogrupper av snutebille-eksemplarer, er støttet av morfologisk data som bekreftet at de 10 gruppene er ulike arter. Noen haplogrupper viser også kryptisk diversitet, der tidligere morfologiske artene viste seg å være unøyaktige. Snutebillenes pollineringsmønster kan indikere spesialiserte pollinerings-interaksjoner med *Araceae* planter, hvor hver haplogruppe ble funnet pollinerende på 1-2 *Araceae* plantearter, til tross for en utilstrekkelig undersøkelse og for liten datamengde til å kunne bekrefte spesialiserte interaksjoner. Denne observasjonen er antatt å delvis bli drevet av reproduksjons-barriere og tilpasning ved evolusjon.

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1. Introduction

1.1 Colombian biodiversity

Colombia with a total area of approximately around 1 150 000 km^2 and currently 75 157 observed species, is considered the second most megadiverse country based on its species richness (Noreña – P et al., 2018). Colombia encompasses a diversity of landscapes, such as the Amazon Forest, the tropical dry forests, páramos, the high Andean forests, and snow-covered mountains. This complex landscape is due to Colombia's geographical context and its geological history (Hermelin, 2016; Llambí et al., 2019).

Most of the slopes of the Andean mountains in Colombia are covered by heavy dense forests, although these forests have been declining due to deforestation to create mountain grasslands and fires (Bates, 1948). The tropical montane rainforest, is known to be the ecosystem with the highest plant diversity in the world, with both temperate and tropical plant species (Cavelier & Tobler, 1998). Within this, the lower montane rainforest, ranges from 1800m to 2400m on tropical mountains (Grubb & Whitmore, 1966). Studies indicate that lower montane forests are associated with less persistent, but frequently low cloud coverage more than a specific temperature range, and can therefore also be referred to as a "cloud forest" (Grubb & Whitmore, 1966; Webster, 2001). Lower montane forests also experience drier periods, where accumulation and replenishment of water are important for biodiversity (Grubb & Whitmore, 1966). The upper part of the lower montane forest (above 2000m altitude) often consists of relatively more moss, and the vegetation here is in general smaller in stature compared to the lowest part of the lower montane forest (Webster, 2001).

Tropical andean forests on mountain slopes, such as lower montane rainforest, are typified by high species richness of epiphytes and herbs, caused by local isolation and a great variety of habitats. The angiosperm family *Araceae* (also called aroids) is one of the three largest plant families of monocotyledons in the neotropics of Northeast South America and is diverse along mountain slopes in the Andes in Colombia (Croat, 1992; Grubb & Whitmore, 1966). Simultaneously, *Araceae* plant richness decreases with elevation and low precipitation (Gómez-Murillo & Cuartas-Hernández, 2016). Even though Colombia is the most species-rich area for *Araceae* plants, this plant family is one of the least studied in the Neotropics

(Croat, 1992). Aroids also have an important structural influence on mountain tropical forests in Colombia (Gómez-Murillo & Cuartas-Hernández, 2016), where Aroids consist of large number of climbers and epiphytes and a large number of interactions with pollinators (Chartier et al., 2014).

1.2 Weevil-aroid pollination system

Plant-pollinator interactions in the tropics are regarded as highly dynamic, according to Gómez-Murillo and Cuartas-Hernández (2016). Furthermore, pollination interactions are influenced by unpredictable changes in the environment, and contribute to ecosystem function and its resilience (Burkle & Alarcón, 2011). A study on climate change effects on plant-pollinator interactions showed reduced floral availability for many pollinators due to phenological shift, which could lead to their extinction (Burkle & Alarcón, 2011). Forest transformation to crop and grazing grasslands in addition to the exploitation of wood, is the main threat to insect biodiversity, according to the regional entity of territorial management (Gómez-Murillo & Cuartas-Hernández, 2016).

Araceae pollination systems have mostly been studied in tropical lowlands. Simultaneously, it is postulated that tropical mountain forests are composed of more complex *Araceae* plant-pollinator interactions due to periods of precipitation and elevation (Gómez-Murillo & Cuartas-Hernández, 2016). Periods of rainfall and high plant species diversity in tropical forests are associated with pollination by animal vectors instead of pollination by wind (Haran, Kergoat, et al., 2023). In general, there has been little attention on weevils as pollinators (Haran, Kergoat, et al., 2023), and they have been considered as poorly adapted generalist pollinators (Eriksson, 1994). However, later studies on pollination have found weevils to be one of the most important specialist pollinators, where a large amount of weevil species are oligophagous (Anderson, 1993; Terry, 2001; Haran, Kergoat, et al., 2023).

Many plant-pollination interactions are mutualistic, including the pollination system of most *Araceae* plants (Chartier et al., 2014). Weevils are one of the insect groups with the specialized pollination system of "brood-site pollination mutualism" (BSPM) (Haran et al., 2022; Haran, Kergoat, et al., 2023). In BSPM the weevil gets trapped, feeds, mates, and

performs oviposition within the aroid plant inflorescence (Eriksson, 1994; Chartier et al., 2014). Weevil species have been observed crawling downwards to the spathe on aroid plants, where they stay protected in the base of the spadix for most of the flowering time without moving much (Franz, 2007; Haran, Kergoat, et al., 2023; Fig. 1), indicating BSPM. Pollen packages are assembled on the weevils during the inflorescence visit and then get transferred when the weevils visit another aroid plant inflorescence (Franz, 2007). Von Martius discovered this weevil-aroid interaction system where weevils were attracted to the inflorescences of the aroid plants as early as 1823 (Franz, 2006). Another study shows a more generalized pollination pattern among weevils (Ødegaard & Frame, 2007). Further, more research on these complex aroid-weevils' pollinating networks is needed.

Figure 1: Inflorescence on the spadix of two Araceae plants, where weevils were found visiting. Pictures: Marthe Svihus.

1.3 Weevil biology

There are approximately 6 000 described genera and more than 62 000 described species in the superfamily *Curculionidea*, known as weevils, in addition to a large number of overlooked species (Marvaldi et al., 2002; Haran, Kergoat, et al., 2023). The superfamily *Curculionidea* consists of seven families*, Nemonychidae, Anthribidae, Belidae, Attelabidae, Caridae, Bentidae,* and the biggest one, *Curculionidae* (Marvaldi et al., 2002), often referred to as true weevils, and known to be the largest animal family regarding species number (Hernández-Vera et al., 2010). Based on both molecular and morphological data, a study claims the

Curculionidae family to be monophyletic, but there have been a lot of systematical changes within the family (Morrone & Marvaldi, 2000; Marvaldi et al., 2002). The most prominent characteristic of adult weevils in the *Curculionidea* superfamily is the "head prolonged into a rostrum", yet the rostrum has large variability in length and shape (Morrone & Marvaldi, 2000). Although there are high similarities between female and male weevils, there are some gender differences within their morphology, where females often have a bigger body size compared to males for example (Rui-Sheng et al., 2021).

Weevil attraction to flowers was first described by von Martius in 1823, when the tribe *Derelomini* weevils (*Coleoptera: Curculionidae: Curculioninae*), later referred to as *Acalyptini*, were suggested to be the first pollinators of some palms. Palaeontological records of *Acalyptini* weevils indicate that these weevils have had an interaction with monocot host plants (*Cyclanthaceae*, *Arecaceae* or *Araceae*) for more than 40 million years (Franz, 2004). The *Acalyptini* weevils have been characterized by reproducing within palms' inflorescence. Recently, there has been more awareness of a more complex pollination pattern, with also undescribed weevil species in addition to described *Acalyptini* weevils. There have been systematic changes with *Acalyptini* and *Derelomini*. Simultaneously, these tribes can be regarded as systematically closely connected (Franz, 2006). Species within the *Acalyptini* tribe were originally a part of the *Erirhininae* subfamily but have later been replaced to the *Curculioninae* subfamily due to 18S ribosomal DNA analysis (Franz, 2006). The last taxonomical studies (including this study) consider the tribe *Derelomini* described by Franz, as the tribe *Acalyptini* described by Thomson (Fig. 2). *Derelomini*, referred to as *Derelomina* by Franz earlier, and *Acalyptina* are now classified as subtribes within *Acalyptini* (Alonso-Zarazaga, 2007). The most prominent characteristic of the *Acalyptini* tribe is the "absence of centrolateral seta on the first (…) and second segments of the maxillary palpi or armed apiced of the tibiae" (Nazarenko & Perkovsky, 2016). The *Acalyptini* tribe consists of 240 recorded species and 41 genera, where most are associated with the inflorescence of *Arecaceae*, in addition to *Araceae* and *Cyclanthaceae* plants (Franz, 2003; Nazarenko & Perkovsky, 2016).

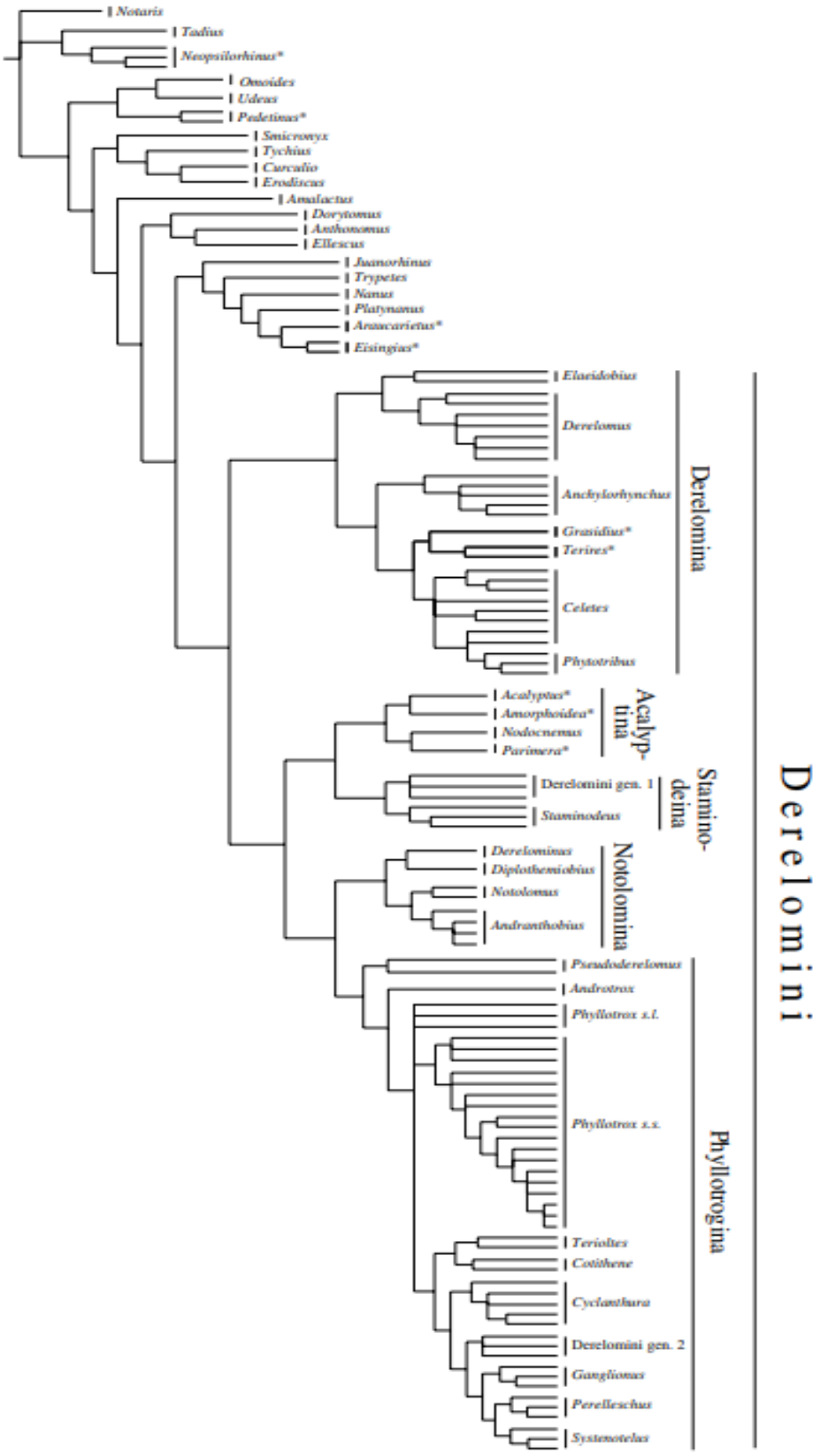


Figure 2: Earlier phylogenetic structure from Franz of the Derelomini (later referred to as Acalyptini) flower weevils, with tribes and subtribes, per 2006. Illustration: Franz, 2006.

The *Acalyptini* tribe consists of five subtribes, *Acalyptina*, *Notolomus*, *Staminiodeina*, *Deremolini,* and *Phyllotrogina*, (Fig. 2). The subtribe *Acalyptina* are mostly pollinators of palms and are distributed in Asia and Australia. *Notolomus* is a North American palm-associated subtribe, while the subtribe *Staminodeina* consists of specialized parasites associated with exclusively *Cyclanthaceae*. The subtribes *Derelomini* and *Phyllotrogina* are found in South America and can therefore potentially be relevant for this study. The subtribe *Derelomini* is described by "rostrum dorsally with multiple (…) subparallel carinae extending (…) from base to subapical region of rostrum" and appears to pollinate inflorescence of palms. The diverse subtribe *Phyllotrogina*, can be considered as taxonomical problematic, that most likely consists of several unknown weevils (Franz, 2006).

A neotropical, diverse and palm-pollinating weevil genus within *Acalyptini* in the *Phyllotrogina* subtribe is *Phyllotrox*. This genus is associated with *Arecaceae and Cyclanthaceae* palms and are therefore not relevant for this study. Later the genus *Cyclanthura* with 15 species, within the *Phyllotrogina* subtribe, was presented within the neotropical *Acalyptini* flower weevils (*Coleoptera: Curculionidae: Curculioninae*) in 2003 by Franz, N. M., based on morphological traits. The *Cyclanthura* genus is associated with the inflorescences of *Araceae* and *Cyclanthaceae,* in addition to being the genus of the morphospecies *Cyclanthura cf. oculata* investigated in this study, which makes this genus relevant for this study. *Cyclanthura* genus can, in general, be identified by "Color (dark) reddish-brown; rostrum in dorsal view fairly narrow, in lateral view ventrally lubricate to slightly arcuate", "Eyes separated by a distance smaller than or similar to breadth of the apex of rostrum" and "Median lobe in dorsal view apically fairly broad, in lateral view apically gradually narrowed". A species within the *Cyclanthura* genus described by Franz, *Cyclanthura oculata*, also the morphospecies investigated in this study, are distinguished mainly by large, protruded eyes among others (2003; Fig. 3). The *Cyclanthura* genus within the *Phyllotrogina* subtribe is highlighted by being mostly associated with *Araceae* plants in South America (Franz, 2003; Franz, 2006). Due to uncertainties within the *Phyllotrogina* subtribe, a lot of taxonomical changes within the *Acalyptin*i tribe, and species only described morphologically within *Cyclanthura*, more certainty in *Acalyptini's* taxonomy by incorporating DNA barcodes would be beneficial.

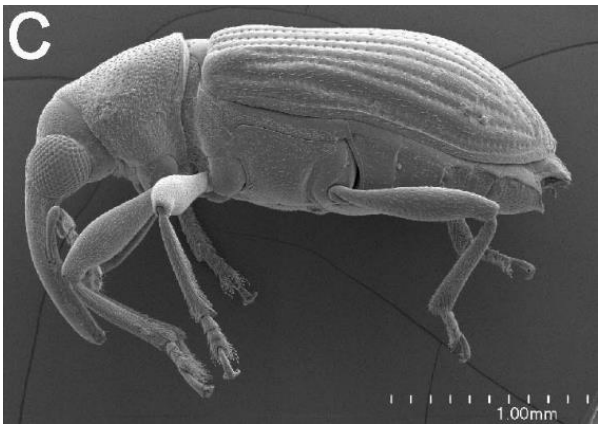


Figure 3: Female Cyclanthura oculata species (Coleoptera, Curculionidae), lateral view, from Fraz, (2003).

Other studies on weevils' systematics, show that weevils within the *Acalyptini* tribe (*Coleoptera; Curculionidae*) are mainly found pollinating palms. Further within the *Acalyptini* tribe, weevils within the *Phyllotogina* genus are found pollinating *Araceae* plants in South America, making this weevil genus interesting for specie identification in this study. However, there are a lot of systematical changes, problematic identifications, and undescribed species within the *Acalyptini* tribe and *Phyllotrogina* genus, thus requiring more research on the systematics of the *Acalyptini* tribe (N. Franz, 2003; N. M. Franz, 2006).

1.4 DNA Barcoding

DNA barcoding as a tool for identification of animal species was first introduced by Hebert et al. (2003) and has been successfully implemented in species identifications for several taxonomic groups (Smith et al., 2005; Kranzfelder et al., 2017; Song et al., 2018). DNA barcoding intends to identify species and discover new species by comparing one or a few short, standardized reference genes (Moritz & Cicero, 2004; Hebert & Gregory, 2005). According to Herbert et al. (2003), DNA barcoding based on the mitochondrial cytochrome c oxidase subunit 1 (COI) sequence is a sustainable and reliable method for taxonomy and biological identifications and this is now a well-established tool in taxonomy (Song et al., 2018). The mitochondrial COI gene is used to a great extent for the identification of insects (Caterino et al., 2000; Armstrong & Ball, 2005). Specifically, the mitochondrial COI gene sequence with its 600-800 bp (Hebert & Gregory, 2005; Rui-Sheng et al., 2021), has been helpful to use in DNA barcoding for most metazoans, especially to identify and separate small, cryptic, and rare species (Kranzfelder et al., 2017).

There are several procedures and algorithms to suggest species boundaries in DNA barcoding, such as distance-based and tree-based methods. The tree-based method is based on species separation as being the smallest diagnosable cluster or being the smallest separately resolving lineage in a phylogenetic tree, while distance-based methods are usually grounded from a genetic similarity threshold value (Song et al., 2018). When using DNA barcoding, it is suggested to have a generalized threshold for the barcode gap around 2-3% in nucleotide composition change in the COI sequences between different species (Hebert et al., 2003; Puillandre et al., 2012), although an optimal threshold value varies among taxon (Lin et al., 2018). This threshold method to distinguish intra- and interspecific divergence does not take into account tree structure (Blair & Bryson Jr., 2017)

A morphospecies is a taxa or group of individuals that is only determined by the organism's morphology, from Taheri et al.'s definition (2018). An idea behind morphospecies as a method for identification has been to let someone without knowledge of taxonomy identify species visually. Morphospecies can then also be described as "recognizable taxonomic units" that have characteristics easy to observe (Derraik et al., 2010). Morphospecies are typically used for species identification in tropical areas, where it is a high biodiversity compared to taxonomists (Derraik et al., 2010) Morphospecies can be considered beneficial for broad taxonomical identifications (Friedheim, 2016). However, species identification based on morphs has limitations such as phenotypic plasticity and cryptic diversity, especially considering that there are millions of species (Hebert et al., 2003; Posada-López et al., 2023). An issue of taxonomy based on morphological descriptions is the lack of common physical characteristics between major groups, which can cause difficulties and disagreements among taxonomists (Friedheim, 2016). According to Krell (2004), the use of morphospecies to identify species does not fulfill the criteria to be considered a scientific identification method. In addition to the use of morphospecies, have weevil species traditionally also been identified by dissection of their genitalia (Davidian & Savitsky, 2017), which can be difficult and time-consuming. Additionally, some weevil species are also separated from each other by larval characteristics (Morrone & Marvaldi, 2000). This leads to the need for other identification methods than only morphological identification, such as DNA barcoding, based on mitochondrial COI sequence (Hebert et al., 2003).

Information about genetic biodiversity, including DNA barcoding is advancing slowly in Colombia (Noreña – P et al., 2018). Less than 5% of all Colombian species are represented in the Biodiversity Information System (BIS) database at a molecular level (Noreña – P et al., 2018). Specifically also within the Curculioninae subfamily, there is a lack of molecular data, causing an inconsistent classification where several tribes have unclear boundaries (Haran, Li, et al., 2023). Genetic diversity in insect species worldwide, based on the mitochondrial COI sequence from the Barcode of Life Consortium database (BOLD), shows a positive correlation with seasonally high-temperature areas (French et al., 2023). More data on genetic biodiversity in Colombia will undoubtedly contribute to better national information on genetic biodiversity, and better identification systems, and facilitate further research on Colombian biodiversity and ecology (Noreña – P et al., 2018).

1.7 Aims of the study

Although the aroid plants are the most species-rich in Colombia and are especially diverse in lower montane rainforests here, there is a lack of knowledge of these plants and their pollination structure. Additionally, field observations by aroid botanists indicate that there is a be a highly specialized and complex BSPM among weevils and aroids. There have also been many uncertainties and knowledge gaps in the taxonomy of neotropical palm-pollinating weevils, due to insufficient identification methods of weevils, which has led to difficulties in understanding the specificity and ecology behind the weevil-aroid pollination system in lower montane rainforests in Colombia. DNA barcoding of aroid-pollinating weevils could be an useful supplement for proper identification of these weevils, and therefore also provide a better understanding of the complex weevil-aroid pollination system in the lower montane rainforest in Colombia.

Given the knowledge gaps regarding: 1) the lack of molecular studies on many weevil genera such as the ones in the neotropical *Acalyptini* tribe, 2) the lack of knowledge or aroid pollination ecology in Colombia and 3) the time-consuming and insufficient weevil identification method relying on morphospecies and weevils' genitalia among others (Haran et al., 2022), the objectives of this studies are:

1) To collect weevils associated with an *Araceae* community in a lower montane rainforest in Colombia and identify these weevil individuals to previous existing morphospecies.
2) To sequence DNA barcodes for weevil species collected based on the mitochondrial COI gene by performing DNA extraction and PCR.
3) Based on the analysis of DNA barcodes, examine the accuracy of the earlier existing morphospecies and possible cryptic diversity within the weevils.
4) Distinguish haplotype groupings of the weevil specimens based on phylogenetic analysis and morphological characteristics.
5) Describe the observed flower-visiting (pollination) pattern for the constructed DNA barcodes for the collected weevils.

2 Method

2.1 Data collection

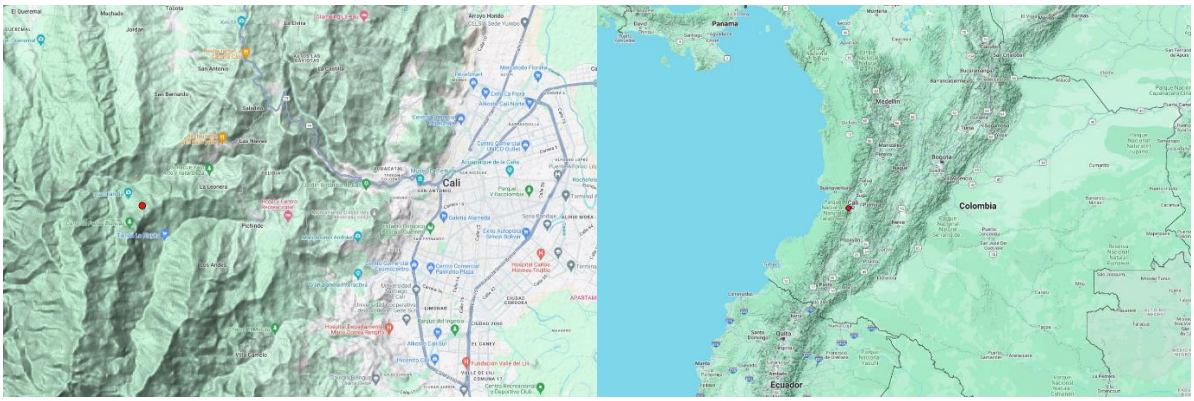


Figure 4: Collection site (red dot) of all specimens included in this project. Illustration: QGIS 3.36.2.

The data for this study was collected in the surrounding forest of Zygia biological station from University ICESI, located in the Cordillera of the Andes in the Department of Valle del Cauca, municipality of Cali, Colombia (Fig. 4; Fig 5). The altitude of Zygia biological station (3.4418°N, 76.6637°W) is 2368m. In total 50 adult weevil specimens (sample codes 1-41, 1B, 2A, 3B, 4C, 4E, 5A, 5F, 6F, and 6I) were collected from this study area, where individuals 1-25 were collected 07.08.2023 and individuals 26-41 were collected 11.08.2023. Specimens 1B, 2A, 3B, 4C, 4E, 5A, 5F, 6F and 6I were collected between 2022 and 2023.

Figure 5: Field position with its vegetation, and collection of weevil specimens to this project. Pictures: Kamal H. Robin and Marthe Svihus.

The weevil specimens were collected from the spadix (inflorescence) of plants from the *Araceae* plant family (Fig. 1). Plant species where weevil individuals were collected were identified in the field by Dr Alejandro Zuluaga Trochez. The weevil individuals 1-41 were stored alive in a fridge (4°C) for approximately 24 hours after collection, before they were stored in 95% ethanol in a freezer (-4°C). Further specimens previously collected by Dr Trochez were included for sequencing - coded 1B, 2A, 3B, 4C, 4E, 5A, 5F, 6F, and 6I were stored in 95% ethanol in a freezer. All weevil specimens were sent from Cali, Colombia to Trondheim, Norway approximately 5 months after collection.

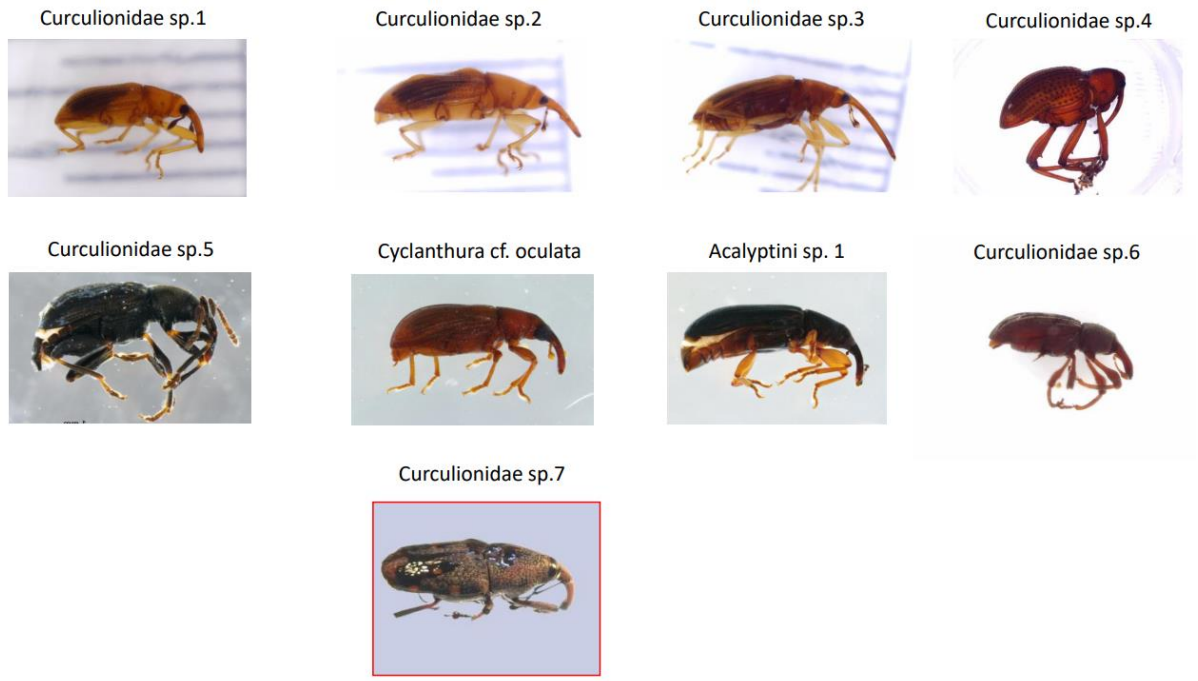


Figure 6: Earlier existing morphospecies within Curculionidae (Coleoptera) from Zygia Biological station, Colombia. Illustration: Allison Muñoz and Alejandro Zuluaga Trochez.

Specimens numbered 1-41 were identified as currently existing morphospecies, used by colleagues at Dr Trochez's institute (Universidad del Valle) (Fig. 6), where the specimens were identified into morphospecies based on this figure (Fig. 6). The specimen's rostrum length and angle were the most crucial factor determining morphospecies. In addition to this, the position of the antennae, antennae color, body color, leg color, and eye size were observed under a dissecting microscope from pictures and recorded. Weevils' exoskeletons were preserved in 70% ethanol after DNA extraction, for approximately two months. Some weevil specimens were then dried and prepared to be included in the NTNU Science Museum,

Department of Natural History's species. Further morphological investigation of specimens in each haplotype group (HG) was done by Frode Ødegaard, to confirm or deny HG as species separation, and to identify specimens within each HGs to the lowest taxonomical level. Identification of the weevils based on their genitalia was not performed.

2.2 Lab methods

2.2.1 DNA extraction

DNA extraction from the weevil specimens was done by using a Qiagen Blood and Tissue kit according to the manufacturer's instructions with a minor modification (see below). The weevil specimens were picked with a needle on one or both sides of their body to pierce their exoskeleton to allow ingress of the tissue lysis (ATL) buffer before being added to the ATL solution for DNA extraction. Sampled were agitated at 390 r.p.m and incubated at 56°C for approximately 8.5 hours. The only modification from the manufacturer's instructions was 100 µl AE buffer added to the columns before incubation to elute the DNA, at the end of the DNA extraction process. The Eppendorf tubes with extracted DNA were stored in a fridge at 4°C until PCR.

2.2.2 PCR

For PCR on all samples, Qiagen Multiplex PCR master mix was used with, the primers: Forward: LCO1490 (5'-GGTCAACAAATCATAAAGATATTGG-3') and Reverse: LCO2198 (5'-TAAACTTCAGGGTGACCAAAAAATCA-3') (Ekrem et al., 2007; Table 1). These primers amplify around 654 bp portion of the mitochondrial COI gene (Ekrem et al., 2007). The standard PCR reaction conditions are provided in Table 1, see Table A1 for more details.

Table 1: Volumes and concentrations of different reagents in one standard PCR sample of 20μl.

Reagent	Concentration	Volume (µl)
Qiagen Multiplex PCR master mix	1x	10
LCO1490 forward primer	0.5µM	1
LCO2198 backward primer	0.5µM	1
Template DNA	-	1
Molecular grade water	-	7

The initial PCR was performed with 1µl DNA extraction solution added to each PCR sample. If PCR amplification failed with this DNA extraction concentration, 5µl or 3µl DNA extraction solution was added to each PCR sample to try to get positive PCR amplification (Table 1A). 1µl BSA was also added additionally to PCR samples where PCR amplification had been failing before (Table 1A). When different DNA template amounts were used, the volume of water was adjusted accordingly to produce a total PCR volume of 20µl (25µl in some PCR samples; Table 1A). PCR thermocycling, after denaturation at 95°C, was first 5-10 cycles with an annealing temperature of 43°C-45°C, then 30-35 cycles with an annealing temperature of 51°C, with extension temperature at 72°C, see Table A2 for the thermocycle programs used for the different samples. See figure 7 for a more detailed description of the PCR. Most of the PCR setups contained one positive and one negative control.

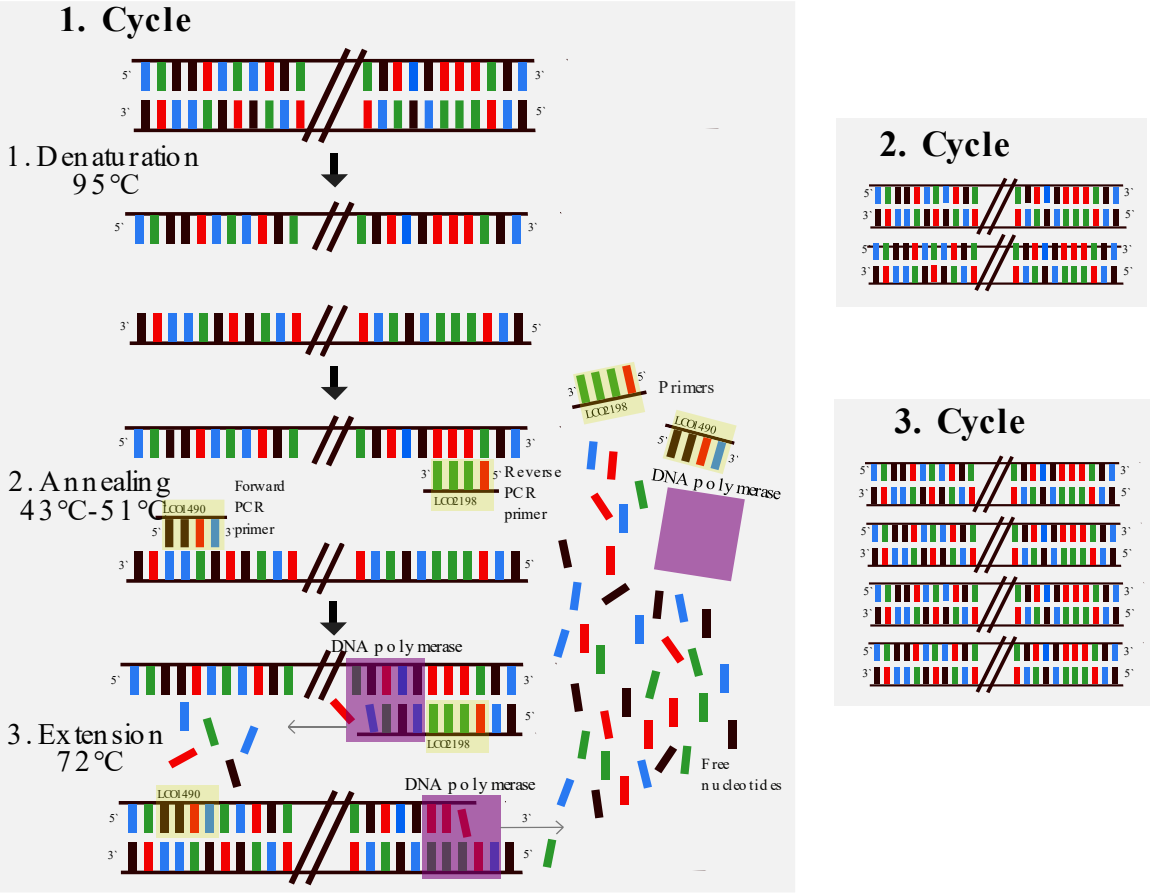


Figure 7: PCR/qPCR done on all samples. LCO1490 and LCO2198 primers were added to the samples, as well as free nucleotides and DNA polymerase from the Qiagen multiplex PCR mastermix. One PCR cycle contains three steps, 1) the denaturation at 95°C which allows denaturing of double stranded DNA strand, 2) the

annealing step at 43℃-51℃ which allows the primers to attach to the DNA strand with the opposite nucleotides and 3) the extension at 72℃ which allows DNA polymerase to attach to the DNA strand with the primers which further facilitates extension of primers to a new opposite DNA strand to the original DNA strand. The extension happens at both strands, one with the forward primer LCO1490 and the other with the reverse primer LCO2198. For every PCR cycle, the number of DNA sequence copies gets doubled. Illustration: Marthe Svihus.

Gel electrophoresis was done on all samples after a PCR to check for positive amplification with the PCR product length. Gel was made with 2% agarose by 1xTAE buffer with SYBR Safe. Electrophoresis on each PCR product, GeneRuler 100bp Ladder, positive control, and negative control, was mixed with 6X TriTrack DNA Loading buffer. Electrophoresis was executed with 45 amperes and 95V for 40 min. Further, a picture of the gel was taken with UV light, using the GeneTools system. Samples with a visible band around 600bp-700bp were considered successful PCR amplification. Samples with successful amplification were purified using Cytive Sera-Mag magnetic beads according to the manufacturer's instructions using a 2.5/1 ratio of beads to PCR. Purified PCR products were sanger-sequenced in both directions by Eurofins genomics.

For unsuccessful PCR samples, a qPCR was performed to monitor amplification in real-time. On these 12 qPCR samples, 0.1 X SYBR Green I (Invitrogen) was added additionally to Qiagen Multiplex PCR master mix, LCO1490 forward primer, LCO2198 backward primer, BSA, and DNA (Table 1A). After initial hold at 95℃ for 5 min, the thermocycling was 10 cycles with annealing temperatures of 43℃ and 45℃, before 30 cycles with annealing temperature of 51℃. At the end it was a melt curve for each qPCR (Table A1; A2). QuantStudio Design and Analysis software was used for analyzing the qPCR results. The addition of SYBR Green to PCRs appeared to have a positive effect on otherwise difficult samples, as most samples appeared to amplify successfully. Due to positive qPCR results for samples with annealing temperatures at both 43℃ and 45℃, qPCR samples with annealing temperatures of 43℃ and 45℃ were combined. The qPCR samples with successful amplification were purified by using the Sera-Mag magnetic beads according to the manufacturer's instructions. Gel electrophoresis was done on the purified samples, the same way it was done on the PCR products, to be sure that the correct DNA segment was amplified after the qPCR. Subsequently, the successful and purified qPCR products were sent to Eurofins genomics for sequencing. Nested PCR and nested qPCR were also tried on unamplified samples, which were unsuccessful.

2.3 Data processing and analysis

For data processing, Seqtrace was used for aligning forward and backward sequences from the same sample, and for trimming the poor quality ends of the DNA sequences. All sequences were manually checked for ambiguities. All DNA sequences were checked in BLAST (NCIB) with the megablast algorithm and in BOLD for the most similar, already registered DNA sequences. MEGA11 software was used for multiple alignment of all DNA sequences, and for creating a phylogenetic tree. Multiple alignments were done by the MUSCLE algorithm, which has a high accuracy, by using distance measures for a pair of sequences (Edgar, 2004). Phylogenetic trees were made by the neighbor-joining method, including bootstrap values from 1000 replicates. The neighbor-joining method used the Kimura 2-parameter (K2P) distance matrix to create phylogenetic trees (Keklik, 2023). The K2P substitution model was used for all phylogenetic analyses. K2P is a method to achieve reliable estimates of the evolutionary distance between two nucleotide sequences by separating transitions from transversions at each site between the two sequences to distinguish the evolutionary distance (Kimura, 1980). Three phylogenetic trees were made in MEGA based only on DNA sequences from this project. One of the trees were based on DNA sequences included in this study, another tree included most similar BLAST DNA sequences, and one tree were based on amino acid translation sequences from this project. Grouped haplotypes based on phylogenetic analysis of DNA sequences and K2P genetic distances from MEGA.

3 Results

In total 42/50 weevil specimens had successful PCR amplification, whereas 41 samples of these also had successful sequencing. The 9 unsuccessful samples are not included in the project any further. The samples 35 and 37 had the least clear sequence in Seqtrace, of the samples considered successful. All trimmed sequences ended up being 649bp-708bp long. The nucleotide frequencies for all sequences in percent were T: 33.7%, C: 20.9%, A: 29.3%, and G:16.1%.

During morphospecies identification of the weevil specimens, all the specimens were identified to 5 out of 9 morphospecies in total (Fig. 6). 11 specimens were identified to *Curculionidae sp. 1*, 5 specimens were identified to *Curculionidae sp. 2*, 7 specimens were identified to *Curculionidae sp. 3*, 14 specimens were identified to *Cyclanthura cf. oculata,* and 3 specimens were identified to *Acalyptini sp. 1*. There were no weevil specimens that were identified as *Curculionidae sp. 4*, *sp. 5* or *sp. 7*.

3.1 Phylogenetic analysis

The phylogenetic distance between all specimens included in this project is visualized by the neighbor joining method (Saitou & Nei, 1987; Fig. 8), where all 41 successful DNA sequences are separated into 10 different haplotype groups (HG). All specimens earlier identified to morphospecies *Cyclanthura cf. oculata* were grouped in HG (HG 1) in the phylogenetic tree (Fig. 8). Specimens from the morphospecies *Curculionidae sp.1*, *sp.2,* and *sp.3* are distributed across several HGs, where the specimens identified to these morphospecies are separated into 5 HGs, then HG 3-HG 7. HG 4 consists of only morphospecies *Curculionidae sp. 3,* HG 6 only consists of morphospecies *Curculionidae sp. 1*, and HGs 3, 5, and 7 consist of at least two *Curculionidae* morphospecies'. The morphospecies *Acalyptini sp.1* have specimens that are separated into two genetically distant groups (HG 8 and 10). There were 3 HGs (HG 2, 8, and 9) that contain only one specimen each (Fig. 8). HG 6 and 7 are sister groups and genetically the two most similar haplotypes. Additionally, there was generally higher intra-haplotype group (intra-HG) genetic variability than inter-haplotype group (inter-HG) genetic variability (Fig. 9).

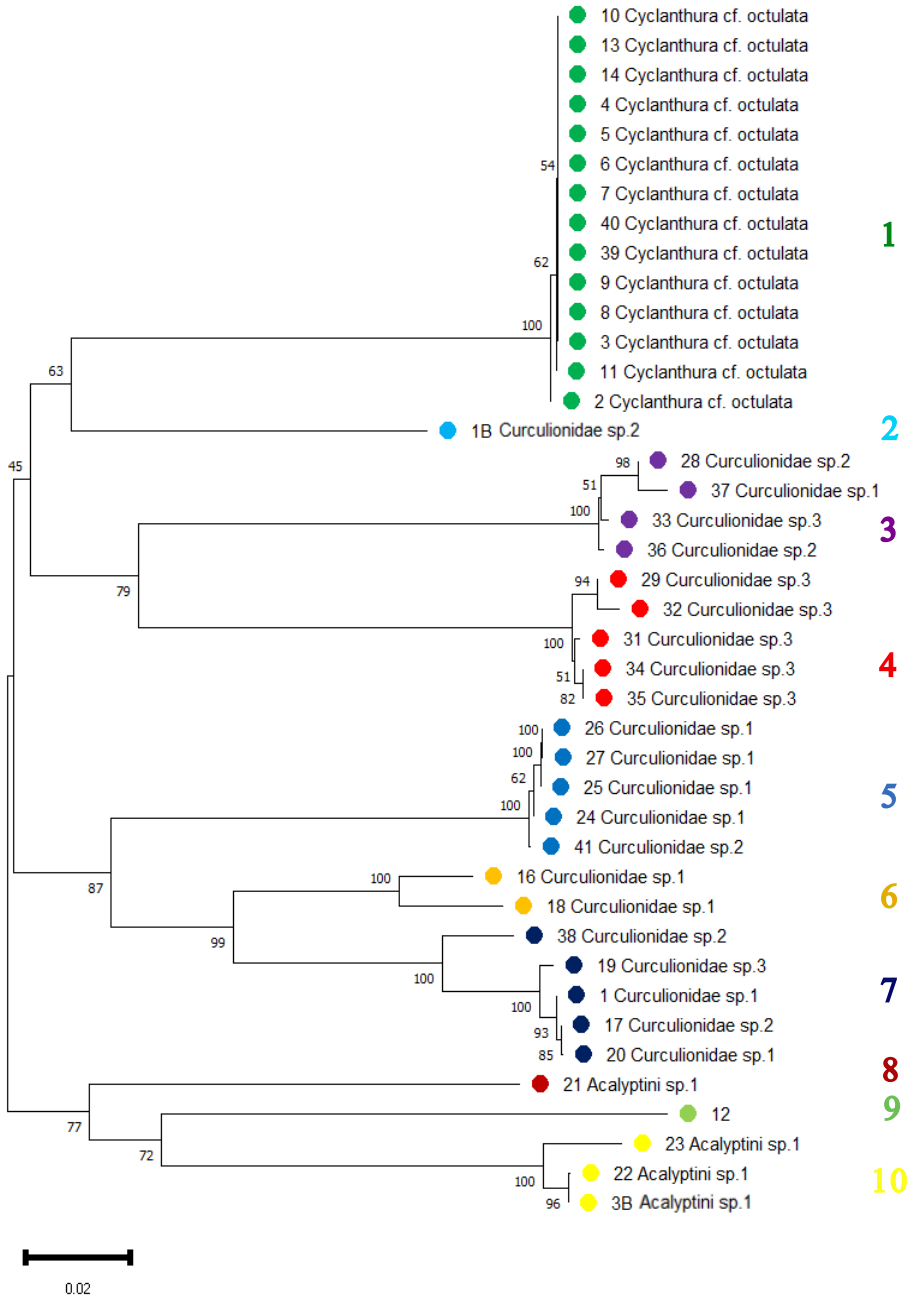


Figure 8: Phylogenetic relationship between all weevil individuals analyzed in this project, all collected in Colombia, conducted in MEGA. HG separation based on the specimens' DNA barcodes shown by distinct colors (dark green:1, turquoise:2, purple:3, red:4, light blue:5, orange:6, dark blue:7, burgundy:8, light green:9, yellow:10). HG separation is decided based on having an intra-HG K2P variance within each HG less than 4% (<0.04 at the scale bar). Marked with earlier identified morphospecies and numbers used in this dataset. Used neighbor-joining tree as statistical method and K2P as substitution model, based on nucleotides. Included bootstrap values to test for phylogeny with 1000 replicates. The scale bar set at 0.02 (equivalent here to 2%) indicates the genetic divergence between specimens.

Intra-HG K2P divergence and inter-HG K2P divergence, based on the HGs (Fig. 8), are shown in (Fig. 9), with standard errors (SE) for each genetic divergence mean. HG 1, where all are identified as morphospecies *Cyclanthura cf. oculata*, shows low intra-HG K2P variance (0.02%, +/- 0.00004) and a high inter-HG K2P divergence (20.83%, +/- 0.00094) with the most specimens sequenced (14). HGs 2, 8, and 9 only consist of one replicate each, making the intra-HG divergence in these HGs irrelevant. HG 6 shows the highest intra-HG K2P divergence of 3.43% (SE not relevant because of only one K2P distance between 2 specimens), also causing the relatively low difference between intra- and inter-HG K2P divergence of 14.43%. The mean difference between the intra- and inter-HG K2P distances (overall) in the HGs is 19.34% (+/- 0.00689), suggesting that it is likely that these HGs represent different species. The intra-HG K2P divergence means lies at 1.15% (+/- 0.00352), where the HGs with only one replicate are excluded (HGs 2, 8, and 9). The average inter-HG K2P divergence overall is 20.15% (+/- 0.00456), and the average inter-HG K2P divergence to the closest HG is 15.98% (+/- 0.00948).

The DNA barcode gap in this project is the difference between intra-HG K2P variance and inter-HG K2P variance overall (Fig. 9), and then the genetic distance to the rest of the DNA sequences included in this project. The DNA barcode gap for HGs with more than one replicate are as follows: 20.82% for HG 1, 20.59% for HG 3, 19.58% for HG 4, 19.94% for HG 5, 14.43% for HG 6, 18.00% for HG 7 and 19.45% for HG 10, (Fig. 9). The mean gap of all the HSs is 19.34% (+/- 0.00689).

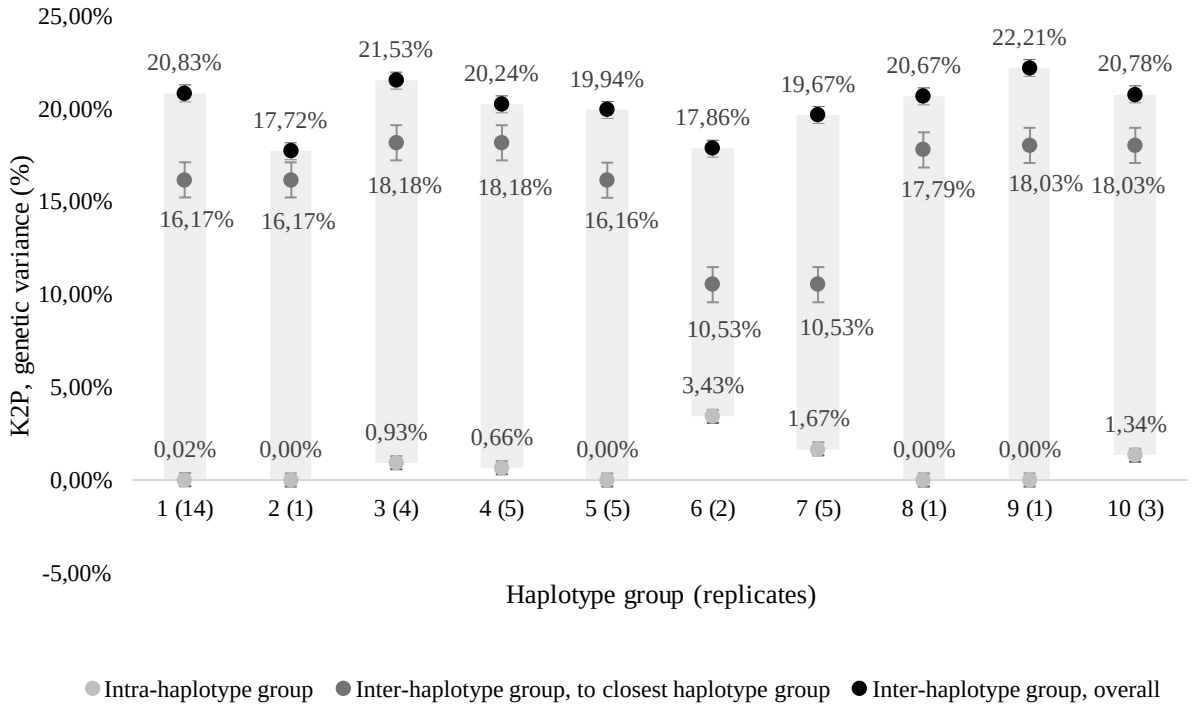


Figure 9: Intra-HG K2P variance (%), inter-HG K2P variance (%) to the lowest genetic distant HG (Fig. 8), and inter-HG K2P variance (%) to all specimens outside the HG, for each HG from figure 6. Genetic variances along the x-axis are means of K2P pairwise genetic variances of nucleotides, conducted from MEGA. Each mean genetic variance value (%) is marked with a standard error bar. Bootstrap tests with 1000 replicates were included in pairwise distance analysis. Conducted in MEGA and Excel.

If all HGs with intra-HG K2P variance higher than 1% (HGs 6, 7 and 10) were split into HGs (HGs 6A, 6B, 7A, 7B, 10A and 10B) such as all intra-HG K2P variance were less than 1% (Table B3), the inter-HG K2P to closest HG became higher than 4% for all split HGs (Fig. B11). Additionally, the split HGs (HGs 6A, 6B, 7A, and 10A) only got one replicate in each HG, which makes the intra-HG K2P variance for these groups not relevant. Moreover, all split HGs got lower inter-HG K2P variance overall than the original HGs (Fig. 9; B11).

All sample sequences showed 81.61%-86.07% similarity to the closest matching COI sequence from BLAST searches, with 99%-100% query coverage (Fig. C12), and an 83.24%-89.39% top match with *Coleoptera*/*Curculionidae* in BOLD. Based on the most similar sequences from BLAST, 8 of these 16 sequences from BLAST, also added to the phylogenetic

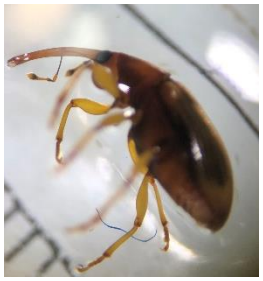
tree (Fig. C12), are registered as individual species in BOLD (Ratnasingham & Hebert, 2007). Phylogenetic K2P distances for the protein-coding amino acid sequences for all specimens (Fig. D13), conducted in MEGA, showed similar phylogenetic relationships between the specimens as the phylogenetic tree based on nucleotides (Fig. 8).

3.2 Haplotype groups

HGs are based on phylogenetic analysis, (Fig. 8; 9) in addition to morphological characteristics (Table 2). The HGs in Table 2 coincide with the HGs from Figure 8. Even though HGs 2, 8, and 9 only have one specimen each, and therefore do not have a relevant intra-HG K2P divergence, the HGs are clearly separated from other HGs (Fig. 9). Morphological characteristics support HGs 2, 8, and 9, where both specimens 21 (in HG 8) and 12 (in HG 9) have distinct characteristics that were not found in any other specimens (Table 2; D4). HGs 1, 4, 8, 9, and 10 have distinctive morphological characteristics not found in other HGs. These characteristics were also seen in all specimens within the HGs, where there was more than one replicate. HGs 3, 5, 6, and 7 have more variable morphological characteristics. In general, it seems like body colour is not a good diagnostic for HG separation, whereas rostrum length and angle are a better diagnostic characteristic for HG separation, especially within HG 1- HG 7, see Table E4 for pictures of all specimens in HGs. HG 1 is identified as *Cyclanthura cf. oculata*, and HGs 2, 3, 4, 5, 6, and 7 are identified as the *Cyclanthura* genus. HGs 8, 9, and 10 are classified as the *Acalyptini* tribe. All the identifications of the HGs were confirmed by F. Ødegaard. The HGs were also all confirmed as being separate species by F. Ødegaard. There are no DNA barcodes for species within the *Acalyptini* tribe from Colombia registered in BOLD, and the genus *Cyclanthura* is not registered in the BOLD system (Ratnasingham & Hebert, 2007).

Table 2: HGs, each numbered 1-10, based on phylogenetic analysis (Fig. 8). Included morphospecies the specimens have been identified to earlier, and which Araceae plant species(except AR 36, AR 37 and AR245), identified by Dr. Trochez, the suggested species were found on during the data collection. The percentage in parenthesis after morphospecies and aroid plants represents the percentage of specimens that fall into a certain category. Each HG has a picture of one of the specimens within a certain HG (from this project), taken within one week after the specimens' collection. Included a description with the most covering and average characteristics and some variable characteristics in grey font, within certain HGs. The taxonomy for each HG shows the identification of the HGs to the lowest taxonomical level possible which applies to all specimens in certain HGs, based on the genetic- and morphological information pointed out in this project. The taxonomy in parathesis is as follows: (order, family, subfamily, tribe, and subtribe) for the HGs.The taxonomical identifications of species and genera of the HGs are done by F. Ødegaard. Pictures of HGs 1 and 3-10 were

taken one day after collection, while a picture of HG 2 was taken more than one year after collection. Pictures: Marthe Svihus.

Haplotype group (specimens)	**Morpho-species**	**Collected from aroid plant**	**Description**	**Taxonomy**	**Picture**
1 (14)	*Cyclanthura cf. oculata* (100%)	*Xanthosoma giraldoi* (50%), *Xanthosoma sanintiae* (50%)	**Characteristics:** Rostrum points down, orange leg color, brown body color, big and protruded eyes.	*Cyclanthura cf. oculata (Coleoptera: Curculionidae: Curculioninae: Acalyptini: Phyllotrogina)*	
2 (1)	*Curculionidae sp. 2 (100%)*		**Characteristics:** Rostrum bends weak.	*Cyclanthura gen. (Coleoptera: Curculionidae: Curculioninae: Acalyptini: Phyllotrogina)*	
3 (4)	*Curculionidae sp. 1* (25%), *Curculionidae sp. 2* (50%), *Curculionidae sp. 3* (25%)	*Stenospermation sp.*	**Characteristics:** Stripes of orange and black on the antenna, rostrum bends weak, yellow legs. Variable body color from brown to orange, and variable body compactness.	*Cyclanthura gen. (Coleoptera: Curculionidae: Curculioninae: Acalyptini: Phyllotrogina)*	
4 (5)	*Curculionidae sp. 3* (100%)	*Stenospermation sp.* (100%)	**Characteristics:** Long rostrum that points out, orange antennae with black clubs, yellow legs. Variable body colors, from orange with black in the center to brown.	*Cyclanthura gen. (Coleoptera: Curculionidae: Curculioninae: Acalyptini: Phyllotrogina)*	
5 (5)	*Curculionidae sp. 1* (80%)	*Anthurium licium* (60%),	**Characteristics:** rostrum bends intermediate, small eyes, orange legs, and black clubs.	*Cyclanthura gen. (Coleoptera: Curculionidae: Curculioninae:*	

	Curculionidae sp. 2 (20%)	*Anthurium croatii* (40%)	Someone with a slightly more compact body, and variable antennae color from orange to brown.	*Acalyptini: Phyllotrogina)*	
6 (2)	*Curculionidae sp. 1* (100%)	*AR36* (50%), *Anthurium angustisectum* (50%)	**Characteristics:** Pronotum and rostrum bend intermediate, small eyes, antennae have stripes of orange and brown. Variable body color.	*Cyclanthura gen. (Coleoptera: Curculionidae: Curculioninae: Acalyptini: Phyllotrogina)*	
7 (5)	*Curculionidae sp. 1* (40%) *Curculionidae sp. 2* (40%) *Curculionidae sp.3* (20%)	*AR 245* (20%), *AR 37* (20%), *A. angustisectum* (40%), *Anthurium croatii* (20%)	**Characteristics:** Rostrum bends intermediate, small eyes. Variable antennae color from orange to brown, someone also with stripes. Variable body color from yellow to brown, often with a black center.	*Cyclanthura gen. (Coleoptera: Curculionidae: Curculioninae: Acalyptini: Phyllotrogina)*	
8 (1)	*Acalyptini sp. 1 (100%)*	*Chlorospatha bullata* (100%)	**Characteristics:** Black body, yellow legs, brown rostrum, and pronotum.	*(Coleoptera: Curculionidae: Curculioninae: Acalyptini)*	

9 (1)	None	*Xanthosoma samatiae* (100%)	**Characteristics:** Black body, pronotum, and legs, brown rostrum, bent pronotum.	*(Coleoptera: Curculionidae: Curculioninae: Acalyptini)*	
10 (3)	*Acalyptini sp. 1*	*Chlorospatha bullata* (100%)	**Characteristics:** Black body, pronotum and rostrum, yellow legs, antennae have stripes of brown and black.	*(Coleoptera: Curculionidae: Curculioninae: Acalyptini)*	

3.3 Weevils-aroid interactions

The number of specimens from different HGs collected from different *Araceae* plant individuals with their species is shown in (Fig. 10). The weevils were collected from 13 different aroid plant individuals, and 10 different aroid plant species. *Stenospermation sp.* (1 specimen) and *C. bullata* (1 specimen) had two different HGs visiting them, where these HG were not found on any other aroid plant species. Several plant species had only one HG visiting, such as *X. giraldoi* (1 specimen), *AR 36* (1 specimen), *AR 245* (1 specimen), *AR 37* (1 specimen), and *A. licium* (2 specimens). No plant species had weevils visiting from more than two different HG. There are no registered plant species for specimens 1B (HG2) and 3B (HG 10) due to earlier collection than specimens 1-41. Note that the sample size for these data is small as there was no extensive effort examining many different individual flowers from the same species.

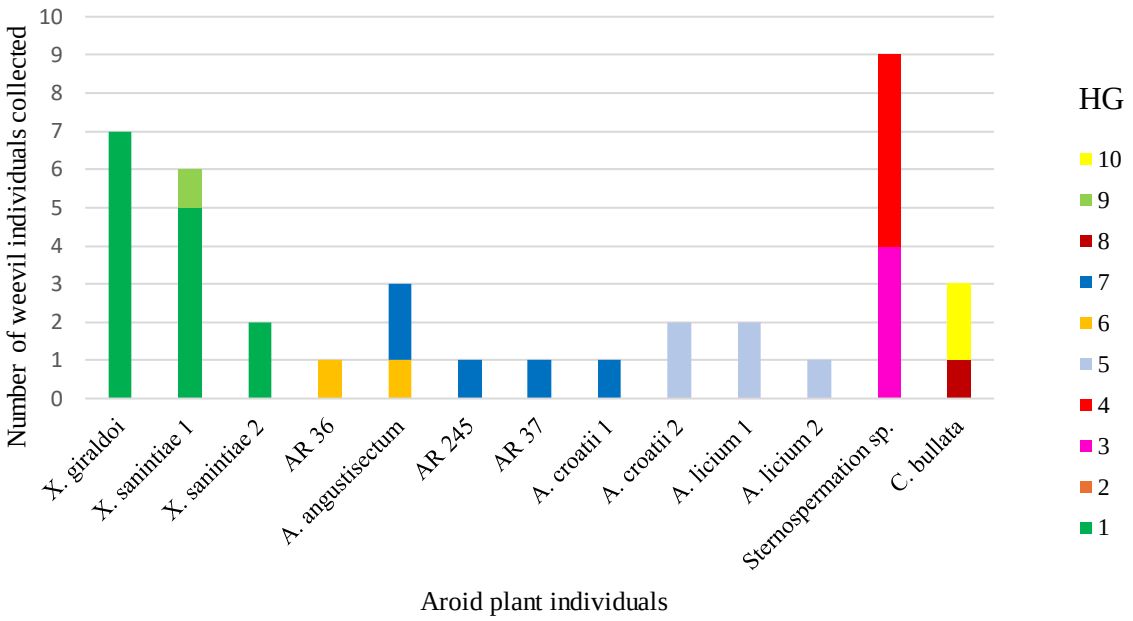


Figure 10: Overview of weevil specimens collected from different aroid plant individuals. Specimens are shown with their suggested species number, based on phylogenetic analysis from Figure 7. Each HG is illustrated with its colour in Figure 7. Aroid plant individuals are listed with their species name. Conducted in Excel.

4 Discussion

The results of this project show the phylogenetic distances, based on DNA barcoding data, between 41 weevil specimens collected at Zygia biological station in Colombia. The 41 successfully amplified and sequenced weevil specimens were distributed into 10 HG, based on molecular analysis. Earlier utilized morphospecies of weevils in Cali, Colombia will be discussed, considering the phylogenetic distances ascertained from this project. Data on aroid-plant visiting for each specimen during collection will also be examined.

4.1 DNA barcoding and taxonomy

DNA barcoding is considered a beneficial species identification method because of the ability to continually add new species, where missing taxa only cause non-identification, not misidentification (Armstrong & Ball, 2005). By refining taxonomic relationships, DNA barcoding contributes to a better understanding of ecology, biodiversity, evolution, and biogeography for conservation (French et al., 2023), since this could reveal greater species richness than species based on morphological traits (Smith et al., 2005). For mtDNA sequences, similar nucleotide composition is often shared between closely related species (Hebert & Gregory, 2005). The COI gene sequence has a higher interspecific divergence than intraspecific divergence, commonly called the “barcode gap”, which makes this gene sequence beneficial for species identifications (Puillandre et al., 2012). This can be seen in general for most of the HGs (Fig. 9). The COI gene has a role in cellular respiration by coding for a respiratory electron transport chain protein (Hill, 2016). Therefore, the mitochondrial COI gene could to some degree be affected by a stabilizing selection which contributes to conserving this gene in all organisms (Hill, 2016). The fact that the COI gene is present in all aerobic organisms, is considered beneficial for its use in DNA barcoding (Smith et al., 2005). Other advantages of using the mitochondrial COI instead of nuclear DNA for DNA barcoding identification are its lack of introns, a low recombination rate, maternal inheritance, and a haploid status (Hebert et al., 2003; Fouda et al., 2022). Mitochondrial COI sequences are also considered useful in DNA barcoding because it has a robust primer supporting recovery of the 5’end of the COI sequence. Additionally, the primer is similar for many species, the COI sequence rarely has insertions and this sequence has a relatively fast molecular evolution rate (Hebert et al., 2003).

However, genetic divergence happens relatively fast in species, which can be an issue for DNA barcoding. Another potential issue of using DNA barcoding in taxonomy is the potential entry of viruses- or parasites DNA through gene transfer (Friedheim, 2016). The weevil specimens' specific age and gender were unknown which also potentially could affect the DNA barcoding process (Ekrem et al., 2007). Due to these potential issues of DNA barcoding, it is crucial to sequence several specimens within the same species to create more certain DNA barcodes for the species, which was achieved in this study in HG's 1, 3, 4, 5, 6, 7 and 10, with respectively 14, 4, 5, 5, 2 and 7 specimens.

Even though DNA barcoding of the COI gene is in general considered convenient and practical, not all the samples in this project were successfully sequenced. 41 samples out of 50 samples were successfully sequenced. Out of the 9 samples unsuccessfully sequenced, 8 samples failed to PCR, with no band observed after gel electrophoresis. A reason for these 8 unsuccessful amplifications, could be the presence of inhibitors (Prieto et al., 2003). Bovine serum albumin (BSA) was added to unsuccessful samples, which reduces the inhibitory effects of various substances (Strien et al., 2013). Other factors that could inhibit PCR amplification in the unsuccessful samples, were CG-rich DNA sequences. Hydrogen bonds are stronger between the nucleotides in CG-regions than in TA-rich regions, which can hinder denaturation of the DNA and primer annealing. However, the CG/TA ratio for all samples in this study was 37/63, which indicates that the DNA sequences are not especially CG-rich, and denaturation failure due to CG-regions is most likely not the reason for unsuccessful samples. Furthermore, it is important to have a specific, optimal annealing temperature to hinder the nonspecific binding of primers. However, different annealing temperatures were tried for the unsuccessful samples. A study claims that low DNA quality could be a more prominent reason for the lack of mtDNA amplification than the presence of inhibitors (Prieto et al., 2003). Furthermore, another study on fungi has shown that specimen storage less than 6 months in 95% ethanol gave successful amplification (Weir & Blackwell, 2001). This could be supported by this study by a biased unsuccessful amplification of the specimens, where 7/8 specimens with unsuccessful amplification were collected before August 2023 and where 40/41 specimens collected in August 2023 had successful amplification. Moreover, the 8 specimens collected before August 2023, were stored for a longer period and therefore might have lower-quality DNA.

One sample sent for sequencing out of 42 samples, had too poor DNA sequencing quality to determine the sequence with certainty. Several other samples also had some uncertainty in their sequencing data. However, having both forward and reverse overlapping sequences for all samples made it manageable to determine the DNA sequences with relative certainty by checking these sequences manually for ambiguities. Clustering of the specimens in HG in addition to long lineages in the phylogenetic tree (Fig. 8), can indicate good-quality DNA barcoding and sequencing. Samples 35 and 37, which had sequence chromatograms with the worst quality of the successful samples, were also clustered in different HG in the phylogenetic tree along with sequences of better quality. This clustering of the poor-quality DNA sequences could be an indicator that, despite some marginal sequencing results, the final edited sequences and the resulting phylogenetic tree results are credible.

The DNA sequences from this study were also examined with BOLD and BLAST search for similarity to other DNA sequences in GenBank. This showed that the most similar DNA sequences from BLAST were 81.61% - 86.07%, and from BOLD were 83.24%-89.39% identical to the sequences from this project, indicating that existing sequences in GenBank were genetically distant to sequences in this project. Comparing the results from BLAST to the DNA sequences in BOLD, only 8 out of the 16 sequences in BLAST are registered as a species (Ratnasingham & Hebert, 2007; Fig. C12). A reason for the long genetic distance to other registered spices, as observed in this study, could be that taxonomically similar weevils from the same area (Cali, Colombia) have not been sequenced before and not because of errors in the DNA sequencing process. Additionally, Franz, N. M. (2006) highlights the lack of molecular data within the *Acalyptini* flower weevils, which could partly explain why the DNA sequences from this study are relatively genetically distant from the already registered DNA sequences in BLAST and BOLD.

The K2P statistical method for calculating the evolutionary distance between two DNA sequences does not consider the codon position in the calculations. This can be considered a weakness of the K2P algorithm when determining genetic distances in this study due to convergent substitutions. Convergent substitutions mean that the codon position where the substitution happens influences the chance that this substitution will cause a drastic change in amino acid properties (Kimura, 1980). Additionally, will an amino acid sequence have an

even higher taxonomic specificity than nucleotide sequences, with more possible amino acids at each position (Bininda-Emonds, 2005)? The fact that the phylogenetic tree based on protein-coding amino acid sequences (Fig. D13), is relatively similar to the phylogenetic tree based on nucleotides (Fig. 8), supports the use of the DNA sequence and phylogenetic distances data (Fig. 8) for the species delimitation.

A standardized and optimal threshold value for intraspecific genetic variation (in this study intra-HG K2P variation) can be difficult to set since the optimal threshold can vary according to taxonomic group and geographic location (Hebert et al., 2003; Puillandre et al., 2012; Lin et al., 2018). The phylogenetic tree in this study shows a large genetic divergence between HGs. The HGs are divided such as the intra-HG K2P distance for each HG is less than 4%, even though an intraspecific threshold value of 2% is common in other DNA barcoding studies (Hebert et al., 2003; Puillandre et al., 2012). Note that there is only one HG where the threshold is more than 3% (HG 6), the rest of the HGs have a threshold of less than 2%. A study done on weevil individuals within the Curculionidae family in China based their species characterization on a 2-3% threshold value (Rui-Sheng et al., 2021). HG 6 should be divided into two distinct groups for possible specie determination, based on a 2-3% threshold value (Fig. 9). In this case with the split HGs, the inter-HG K2P variance to the closest HG is between 2% and 3.73%, which can be evaluated as relatively low (Fig. B11). If all the HG with a threshold of more than 1% were split, most of the split HGs (HGs 6A, 6B, 7A, and 10A) only have one specimen each, contributing to a weaker foundation to claim that these smaller groups could be separate species. Considering this, an intra-HG K2P threshold value of 4% has been used in this study, rather than 2% to include HG 6 as one group. However, DNA barcoding by using the threshold method and/or phylogenetic analysis in itself is not sufficient to discover new species, morphological analysis or information about natural history is also required for creating species boundaries (Hebert & Gregory, 2005; Puillandre et al., 2012). The phylogenetic tree for the HGs 6 and 7 shows how species separation based on only genetical data is not necessarily straightforward, where these HG do not show separated lineages and groups (Fig. 8). This means that the determination of species boundaries within these specimens should include morphological data additionally. Morphological identifications done by F. Ødegaard support that the HGs based on a 4% threshold value (Fig. 8) represent different biological species. This further indicates that a fixed, common threshold value for species separation for different taxa, does not necessarily work. In this study, specie

separation based on the HGs from a 4% threshold value can be considered relatively clear, since the mean inter-HG K2P distance overall is 20.15% (+/- 0.00456; Fig. 9). A high gap between intra-HG K2P variance (threshold value) and inter-HG K2P variance as this study shows where the mean gap for all the HG is 19.34% (+/-0.00689), is considered as beneficial for specie separation.

For valid and good species identification, both morphological and molecular data are needed (Hebert & Gregory, 2005). The species separation of HG 2, 8, and 9 is supported by having morphologically distinct and noticeable characteristics (Table 2), even though there is only one specimen in each HG. In this case, the species separation is inspired by the tree-based method since all these HGs have a separate lineage in the phylogenetic tree (Song et al., 2018; Fig. 8), which supports this suggested species boundary. Even though the specimens in both HGs 8 and 10 were morphologically identified to the morphospecies *Acalyptini sp. 1*. The specimens in HG 10 have different morphological characteristics by being black-coloured except at the legs, compared to specimen 21 in group 8, by having brown color at the head, among others (Table 2). This additionally supports the species separation of HGs 8 and 10. By briefly comparing the morphology of HGs 8 and 10 with the morphospecies *Acalyptini sp.1* (Fig. 6), the specimens in HG 10 seem to have the most similar morphological characteristics with morphospecies *Acalyptini sp. 1*. This could indicate that the specimens in the HG 10 could be identified to the morphospecies *Acalyptini sp. 1*. This would also correspond with the taxonomical identification of HG 10 to the *Acalyptini* tribe. Although, more research within the *Acalyptini* tribe and morphological investigation of HG 8, 9, and 10 are needed to identify these HG to a lower taxonomical level, and to confirm or deny HG 10 being morphospecies *Acalyptini sp.* 1.

A morphospecies can be considered a good identification method if all specimens within one HG are identified as the same morphospecies, where also all specimens identified to one morphospecies are grouped in one HG (Table 2). This is the case with HG 1 where all specimens were identified to *Cyclanthura cf. oculata*. Additionally, since all specimens in HG 1 are identified as the morphospecies *Cyclanthura cf. oculata*, this morphospecies can be considered accurate for species identification. HGs 4 and 10 also have all specimens identified to the same morphospecies, in addition to having relatively distinct characteristics

with respectively a long rostrum that points out (HG 4), and being black except having yellow-colored legs (HG 10; Table 2). However, in this case as well, other weevils not collected in this study could be morphologically very similar to these HGs. More research and investigation are therefore needed here to get a greater foundation to claim that morphospecies work well for identification by discovering more *Araceae* pollinating weevils. Even though identification by morphospecies as observable and "recognizable taxonomic units", is a quick way to identify species, the accuracy of the identification varies a lot among taxa, according to a study on arthropods (Derraik et al., 2010). This is also supported by this study, where specimens identified to the morphospecies *Curculionidae sp. 1*, *sp. 2,* and *sp. 3*, are in general spread out to different HGs (Fig. 8), where specimens in HGs 3, 5, 6, and 7 has various characteristics hard to distinguish (Table E4). This leads to morphospecies Curculionidae sp. 1, 2, and 3 being inaccurate for species identification (Table 2), due to cryptic diversity within these morphospecies. In general, more research is needed to improve the morphospecies identification system of aroid-pollinating weevils.

Since the phylogenetic distance from the sequences in this project to other registered species in BLAST and BOLD is relatively large (Fig. C12), the specimens from this project could be already existing species described morphologically and not genetically, as turns out to be the case for HG 1 identified to *Cyclanthura cf. oculata*. HG 1 shows a large inter-HG K2P variance gap (20.82%), in addition to having a low intra-HG K2P distance (0.02%) and a relatively high number of individual specimens sequenced (14 specimens; Fig. 9). . Morphological characteristics only found on the specimens within HG 1, also supported this species separation, where all these specimens had large, protruded eyes for instance (Table 2; E4). Large, protruded eyes are also a distinct characteristic of the described species *Cyclanthura cf. oculata* (Franz, 2003; Fig. 3). Based on this, it is reliable to assume that HG 1 is the morphospecies *Cyclanthura cf. oculata* (Fig. 6), also described by Franz (2003), since also all specimens identified to *Cyclanthura cf. oculata* are clustered together in the phylogenetic tree (2003; Fig. 8). This coincides also with specie identification of HG 1 done by F. Ødegaard. The low intra-HG K2P divergence then indicates that the morphospecie *Cyclanthura cf. oculata* for the time being, does not have any cryptic diversity.

In contrast to HG 1, the HGs 2-10 were not identified to species level. Hence, as all HGs 2-7 are identified to the genus *Cyclanthura*, these HGs could either be a species morphologically described by Franz (2003) or an undescribed species within the *Cyclanthura* genus, since the *Cyclanthura* genus is either not registered in BOLD (Ratnasingham & Hebert, 2007). To be able to identify other HGs (HG 2 – HG 7) in a species within *Cyclanthura* based on Franz's description (2003), the weevils' genitalia had to be dissected and investigated, which was not done within this study. Therefore, further investigation of these weevils' genitalia is needed to potentially determine the HG 2-HG 7 to a morphologically described species by Franz (2003). HGs 8, 9, and 10 were only identified to the *Acalyptini* tribe by F. Ødegaard, although these three species are most likely within the *Deremolini* or *Phyllotrogina* subtribe, considering that these subtribes consist of South American *Araceae* pollinating weevils (Franz, 2006). Additionally, further research and morphological investigations of these species (HGs 8, 9, and 10) are needed to identify these weevils to genera and/or species level.

4.2 Pollination pattern

The genetic diversification and cryptic diversity of the weevil specimens *(Coleoptera: Curculionidae*; Fig. 8*)* could potentially come from a specialized weevil-*Araceae* pollination interaction. Data from this project could support a specialized and nested pollinating pattern since the specimens from each HG have been collected from only 1 or 2 different *Araceae* plant species (Fig. 10). However, the sample size in the study is too small to draw robust conclusions. A nested or asymmetrical weevil-aroid plant interaction network can relate to a mating hierarchy (Burkle & Alarcón, 2011), where mating of individuals visiting the same host plant happens more frequently than weevils visiting different host plants (Hernández-Vera et al., 2010). Pollinating weevils that have reproductive barriers as described, can be a reason for a higher genetic similarity for weevils visiting the same plant (Hernández-Vera et al., 2010).

Several studies have indicated a specialized weevil-aroid BSPM (Anderson, 1993; Terry, 2001; Haran, Kergoat, et al., 2023). However, another study claims that the genus *Cyclanthura* has the potential to visit and colonize several different *Araceae* plants with different inflorescence biology (Franz & Valente, 2006). Also, a large sample study showed a high number of species visiting two different plant hosts (Ødegaard & Frame, 2007).

Although, this study seems to support a specialized pollination pattern within the Cyclanthura genus, since HG 1-HG 7 (36 specimens), all identified as separate species within the *Cyclanthura* genus, are asymmetrically collected from 8 different aroid plant species (Fig. 10). Although more research with a bigger sample size is needed to confirm this. Moreover, Salzman et al. (2021) show that closely related weevil species are attracted to different chemicals produced by specific host plants in BSPM, which cause specialization and diversity. These different results in pollination diversification and specialization would be interesting for further research on *Cyclanthura*'s pollination pattern.

Plant-insect pollination interactions influence the morphology of both the plant and the insect, and the weevil-*Araceae* pollination interactions are no exception. Therefore, physiological, and morphological adaptation through evolution to enable effective pollination on specific plants could contribute to a specialized aroid-weevil interaction pattern (Chartier et al., 2014). According to Franz (2004), co-evolution between *Acalyptini* weevils and host plants has happened over a long time, which could contribute to a highly specialized and diverse feeding and mating pattern. A study has connected the evolution of long mouthparts in pollinators to long-tubed flowers (Whittall & Hodges, 2007). Despite a small sample size, in this study, HGs 4 (4 specimens) and 5 (5 specimens) seem to have the longest mouthparts (rostrum) of all specimens within this study (Table E4), and they were also collected from the same aroid specie. This may suggest a possible co-evolution as described earlier (Franz, 2004; Chartier et al. 2014). However, future research with a bigger sample size that also considers the plant morphology is necessary to make any conclusions about a potential co-evolution within the *Cyclanthura*-aroid pollination system.

This study could potentially indicate a relationship between genetic distances and pollination specialization, supporting previous studies finding that a niche shift in plant-pollinator interactions leads to the diversification of weevils (Marvaldi et al., 2002). The HGs with less clear and separated phylogenetic lineages, HGs 6 (2 specimens) and 7 (5 specimens), were collected from respectively 2 and 4 different aroid plants. While HGs 3 (4 specimens), 4 (5 specimens), 8 (1 specimen), and 10 (3 specimens) showed more clear and separated genetic lineages and were all collected from one aroid species and individual (Fig. 8; 10). Due to the small sample size in this study, the suggested connection between *Acalyptini*'s genetic

similarity and aroid host plan plant, supported by previous studies would be interesting for further research.

Endemism is high for *Araceae* plants in the Andean mountains at the middle elevation (Croat, 1992), which could contribute to a specialized pollination pattern that only can be found in these mountains. Some HGs in this project (or other aroid-pollinating weevils in lower montane rainforests in Colombia) could be endemic, due to high endemism within the *Araceae* plant family and a potential high weevil pollinating specialization to the aroid plants in the lower montane rainforest in Colombia. A potentially high, complex, and specific weevil–aroid pollination pattern by BSPM (Anderson, 1993; Terry, 2001; Haran et al., 2022; Haran, Kergoat, et al., 2023, could also cause a large genetic diversity within these flower weevils since aroid plants also are considered abundant at mountain slopes in the Andes (Grubb & Whitmore, 1966; Croat, 1992). A complex and specialized plant-pollination network can additionally include inter-annual variability in plant-pollinator interactions where some pollinators are not able to interact with each other due to different timing in pollination (Burkle & Alarcón, 2011). This could potentially mean that there exists a larger diversity of pollinating weevils than was possible to investigate within this project, due to the timing of collection.

Intraspecific genetic variation (Intra-HG K2P in this study) is fundamental for evolutionary change, and thus also for biodiversity. Global climate change also affects genetic diversity, by impacting species demographic distribution which could for instance change the pollination pattern of weevils. In addition, climate change by environmental stochasticity could affect species' phenotypic plasticity and species' evolutionary adaptation to a rapidly changing environment (Pauls et al., 2013). Especially considering the effect of climate change, understanding and conservation of genetic diversity can be considered crucial to maintaining resistant weevil populations. Specifically small- and medium-sized pollinating beetles, as investigated in this study, are shown to be highly important in the maintenance of forest ecosystem function and species biodiversity (Ødegaard & Frame, 2007).

5 Conclusions

The phylogenetic distances between aroid-visiting weevils conducted in this project by DNA barcoding clearly show that there is cryptic diversity among weevil specimens in relation to the currently used morphospecies. This cryptic diversity observed has led to difficulties in identifying *Araceae* pollinating weevil morphospecies based on external morphological traits. The phylogenetic analysis conducted in this project has resulted in 10 clear, separate HGs, confirmed also morphologically being separate species, with a high gap between intra- and inter-HG K2P divergence. Based on this study, DNA barcodes for earlier morphologically described species *Cyclanthura cf. oculata* have shown to be its own separate species and its field identifications appear valid. In contrast, further investigation of weevils' morphological characteristics, such as their genitalia, is needed to investigate the 9 unidentified *Acalyptini* species presented in this study, whereas 7 of these are identified to the *Cyclanthura* genus – these may represent previously described species, or possibly undescribed species new to science. The clearly, separate HGs observed after this phylogenetic analysis, could indicate that the weevil's evolutionary history has been affected by their ecological interaction with aroid plants, potentially by adaptive evolution and reproductive barriers. Although our survey effort was minimal and sample sizes small, the weevil-aroid interaction pattern observed in this project is according to previous studies, by supporting a specialized pollination pattern among *Cyclanthura* weevil species.

This study confirms that there exists an enormous undiscovered biodiversity both at the genetic and species levels in the flower weevils of the lower montane rainforest in Colombia. Since there is a lack of genetic information in Colombia, more studies on phylogenetic analysis and barcoding would be beneficial for easier and more certain species identification. More molecular studies would also improve the understanding of Colombian weevil biodiversity, including genetic and cryptic diversity. More studies on pollination interactions in a lower montane rainforest are needed to get a better understanding of the complex and specialized pollination weevil-aroid pollination system, which is highly needed for conservation of weevils, aroids, and the lower montane rainforest biodiversity in general.

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Appendix A

Table A1: Lab method variations in all samples with successful amplification. Specific thermocycle programs (A-D) are shown in Table A. 2.

Sample	Type of PCR	Thermocycle program	BSA	SYBR	DNA	PCR volume
1	qPCR	C+D	0.2µl	0.4µl	3µl	20µl
2	PCR	A	-	-	1µl	20µl
3	PCR	B	-	-	1µl	25µl
4	PCR	B	-	-	1µl	25µl
5	PCR	B	-	-	1µl	25µl
6	PCR	B	-	-	1µl	25µl
7	PCR	B	-	-	1µl	25µl
8	PCR	B	-	-	1µl	25µl
9	PCR	B	-	-	1µl	25µl
10	PCR	A	-	-	1µl	20µl
11	PCR	B	-	-	1µl	25µl
12	PCR	B	-	-	1µl	25µl
13	PCR	B	-	-	1µl	25µl
14	PCR	B	-	-	1µl	25µl
15	PCR	B	-	-	1µl	25µl
16	PCR	A	-	-	1µl	20µl
17	qPCR	C+D	0.2µl	0.4µl	3µl	20µl
18	PCR	A	-	-	1µl	20µl
19	qPCR	C+D	0.2µl	0.4µl	3µl	20µl
20	qPCR	C+D	0.2µl	0.4µl	3µl	20µl
21	PCR	B	-	-	1µl	25µl
22	PCR	A	-	-	1µl	20µl
23	qPCR	C+D	0.2µl	0.4µl	3µl	20µl
24	PCR	B	-	-	1µl	25µl
25	qPCR	C+D	0.2µl	0.4µl	3µl	20µl
26	qPCR	C+D	0.2µl	0.4µl	3µl	20µl

27	qPCR	C+D	0.2µl	0.4µl	3µl	20µl
28	PCR	B	0.2µl	-	5µl	20µl
29	PCR	B	-	-	1µl	25µl
31	PCR	B	-	-	1µl	25µl
32	PCR	B	-	-	1µl	25µl
33	PCR	A	-	-	1µl	20µl
34	PCR	B	0.2µl	-	5µl	25µl
35	qPCR	C+D	0.2µl	0.4µl	3µl	20µl
36	PCR	A	-	-	1µl	20µl
37	qPCR	C+D	0.2µl	0.4µl	3µl	20µl
38	PCR	B	-	-	1µl	25µl
39	PCR	B	0.2µl	-	5µl	20µl
40	PCR	A	-	-	1µl	20µl
41	qPCR	C+D	0.2µl	0.4µl	3µl	20µl
1B	PCR	B	0.2µl	-	1µl	25µl
3B	qPCR	C+D	0.2µl	0.4µl	3µl	20µl

Table A2: Thermocycling programs A-D used in PCR and qPCR.

A

	Temperature	**Time (min: sec)**	**Cycles**
Stage 1	95°C	5:00	1x
Stage 2	95°C	0:30	5x
	45°C	0:30	
	72°C	1:00	
Stage 3	95°C	0:30	35x
	51°C	0:30	
	72°C	1:00	
Stage 4	72°C	5:00	1x
	4°C	∞	

B

	Temperature	**Time (min: sec)**	**Cycles**
Stage 1	95°C	5:00	1x
Stage 2	95°C	0:30	5x
	45°C	0:30	
	72°C	1:00	

Stage 3	95℃	0:30	35x
	51℃	0:30	
	72℃	1:30	
Stage 4	72℃	5:00	1x
	4℃	∞	

C

	Temperature	**Time (min: sec)**	**Cycles**
Stage 1	95℃	5:00	1x
Stage 2	95℃	0:30	10x
	45℃	0:30	
	72℃	1:00	
Stage 3	95℃	0:30	30x
	51℃	0:30	
	72℃	1:30	
Melt curve	72℃	5:01	1x
	95℃	0:01	
	60℃	0:20	
	95℃	0:01	

D

	Temperature	**Time (min: sec)**	**Cycles**
Stage 1	95℃	5:00	1x
Stage 2	95℃	0:30	10x
	43℃	0:30	
	72℃	1:00	
Stage 3	95℃	0:30	30x
	51℃	0:30	
	72℃	1:30	
Melt curve	72℃	5:00	1x
	95℃	0:01	
	60℃	0:20	
	95℃	0:01	

Appendix B

Table B3: Specimens distributed in new groups, used in Figure B 11, where all intra-HG K2P distances are less than 1%.

New groups	Specimens
6A	16
6B	18
7A	38
7B	1, 17, 19, 20
10A	23
10B	22, 3B

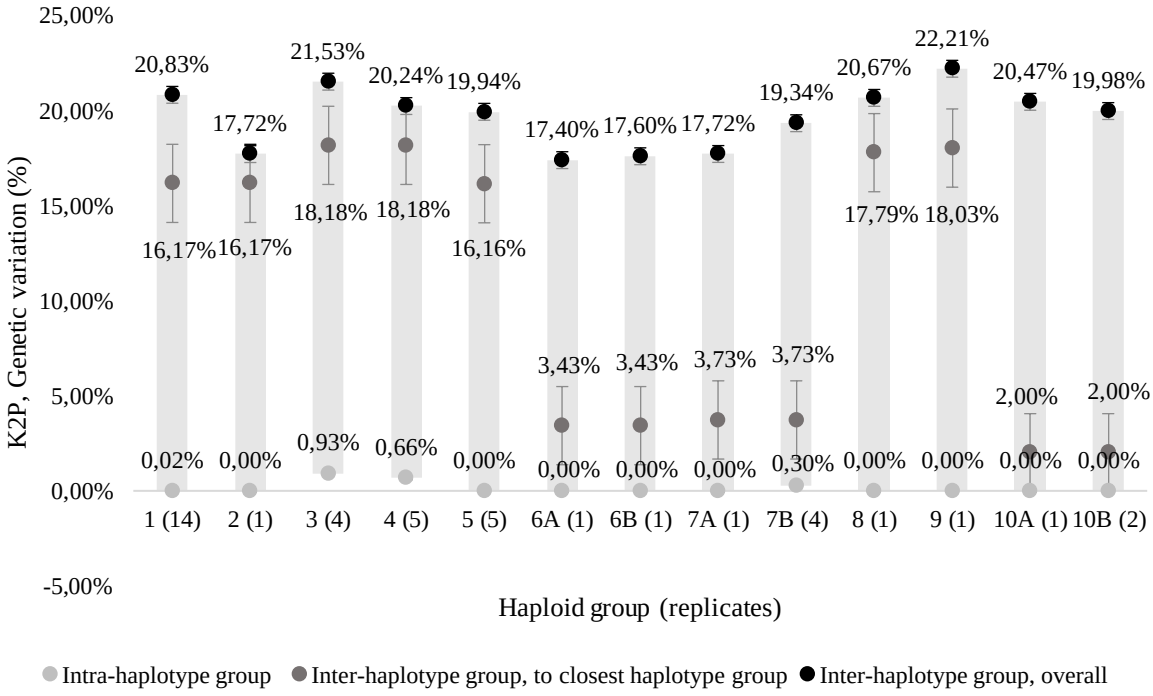


Figure B11: Intra-HG K2P variance (%), inter-HG K2P variance (%) to the lowest genetic distant HG (Fig. 6), and inter-HG K2P variance (%) to all specimens outside the HG, for each HG (Fig. 6). HGs 6, 7, and 10 are split into smaller HGs (Table B3). K2P genetic variances are based on pairwise distance means of nucleotides with the K2P model, carried out from MEGA. Each mean genetic variance value (%) are marked with a standard error bar. Bootstrap tests with 1000 replicates were included in pairwise distance analysis. Conducted in MEGA and Excel.

Appendix C

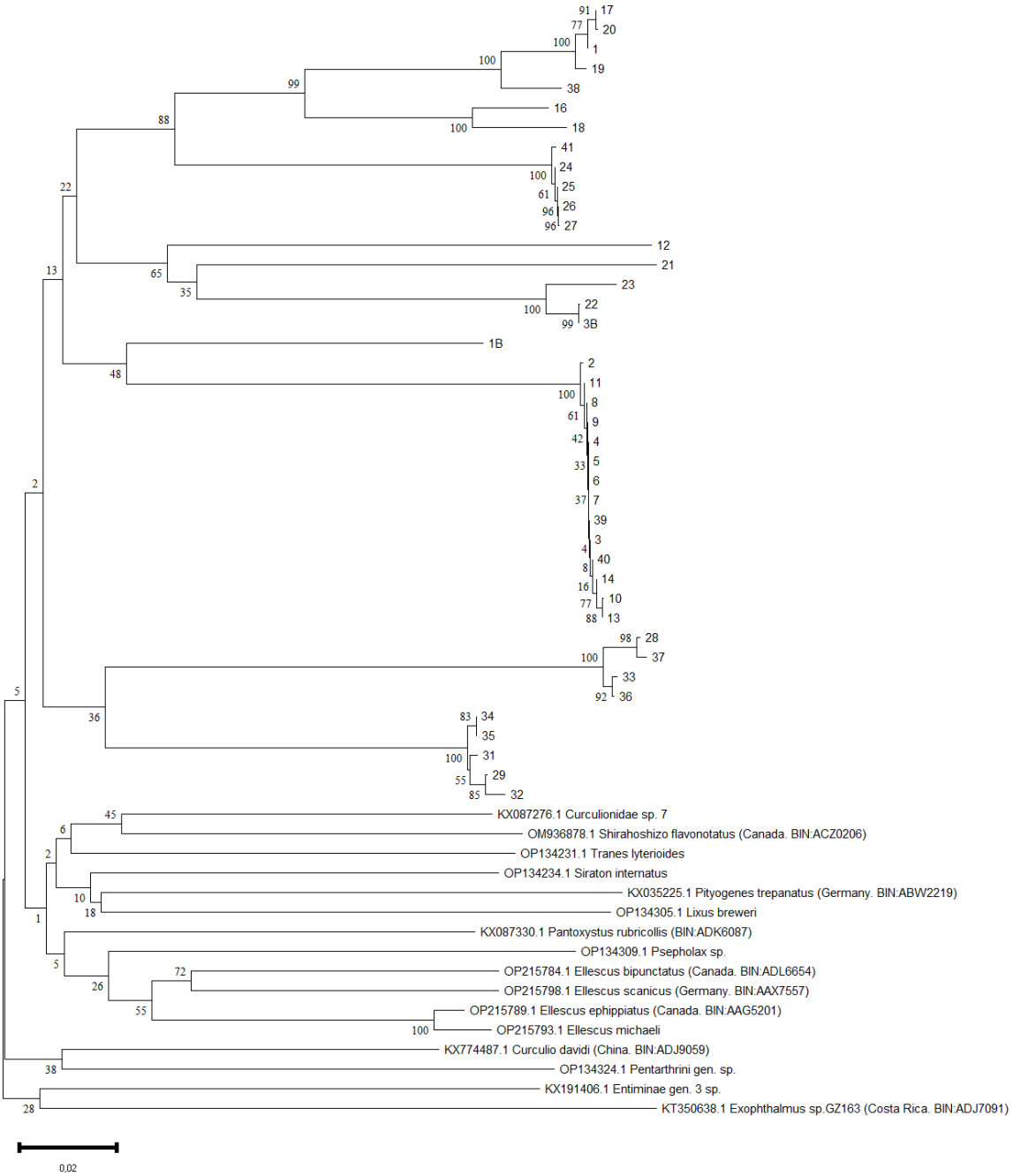


Figure C12: Phylogenetic distances between DNA sequences from specimens within this project marked with earlier identified morphospecies and species number used in this dataset (1-41,1B,3B) and the most similar sequences registered to these. Obtained from NCBI BLAST (megablast) with 99%-100% query coverage and 81.61%-86.07% identical DNA sequence to at least one of the DNA sequences from this project. 1-3 BLAST sequences were added for each specimen, depending on the query coverage, where some BLAST sequences were overlapping with several specimens. Added in total 16 BLAST sequences in addition to the DNA sequences from the specimens in this study. Every species DNA sequence obtained from BLAST is enrolled with sequence ID from BLAST followed by species name. Registered species in BOLD are indexed with collection site and BIN within parenthesis, sourced from BOLD. Used neighbor-joining tree as statistical method and K2P as substitution model, based on nucleotides. Included bootstrap values to test for phylogeny with 1000 replicates. The scale bar set at 0.02 indicates the genetic divergence between specimens and species.

Appendix D

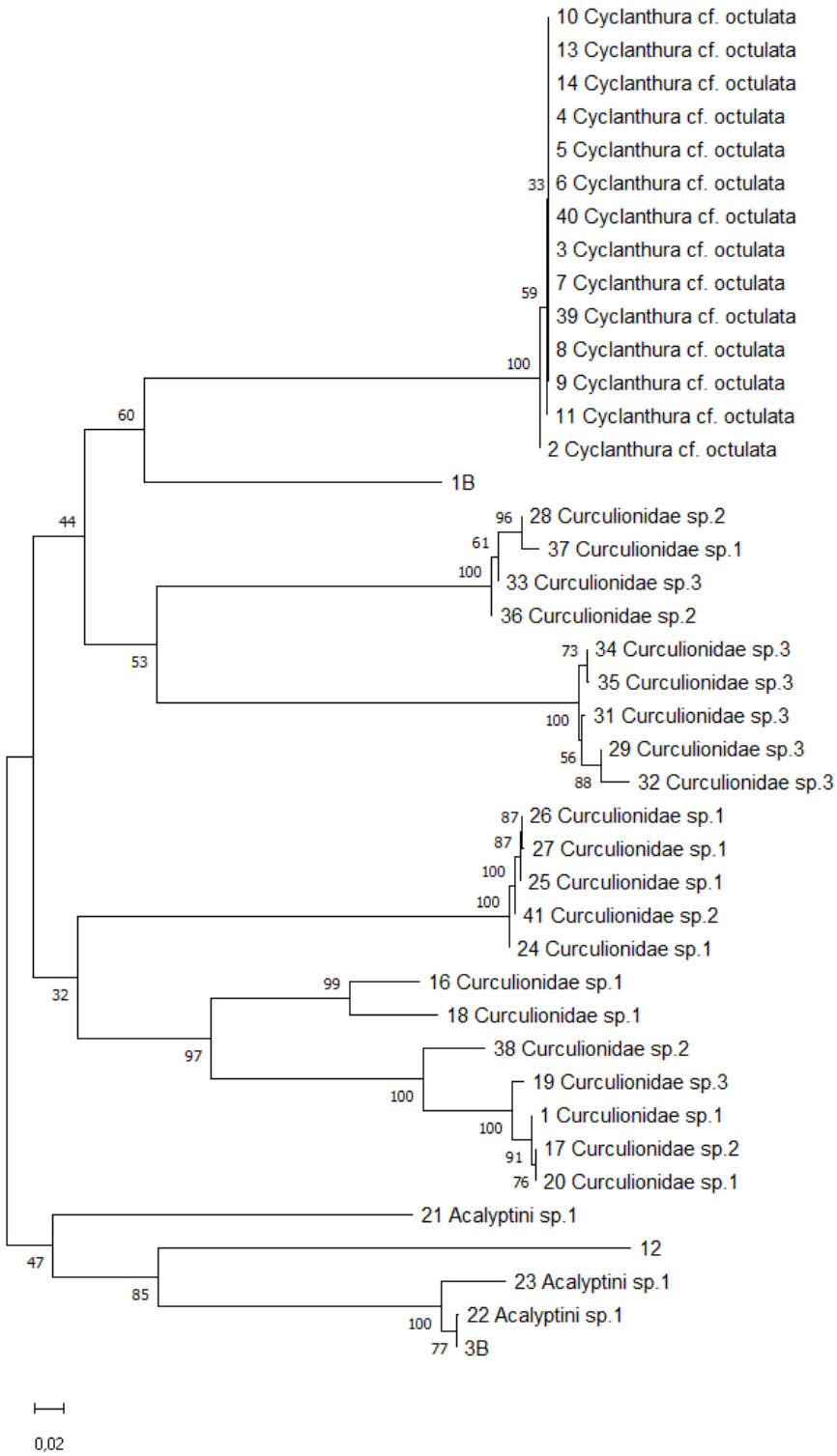
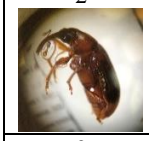
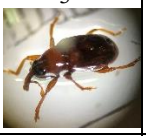
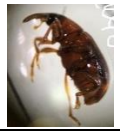
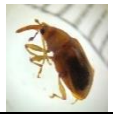
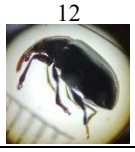
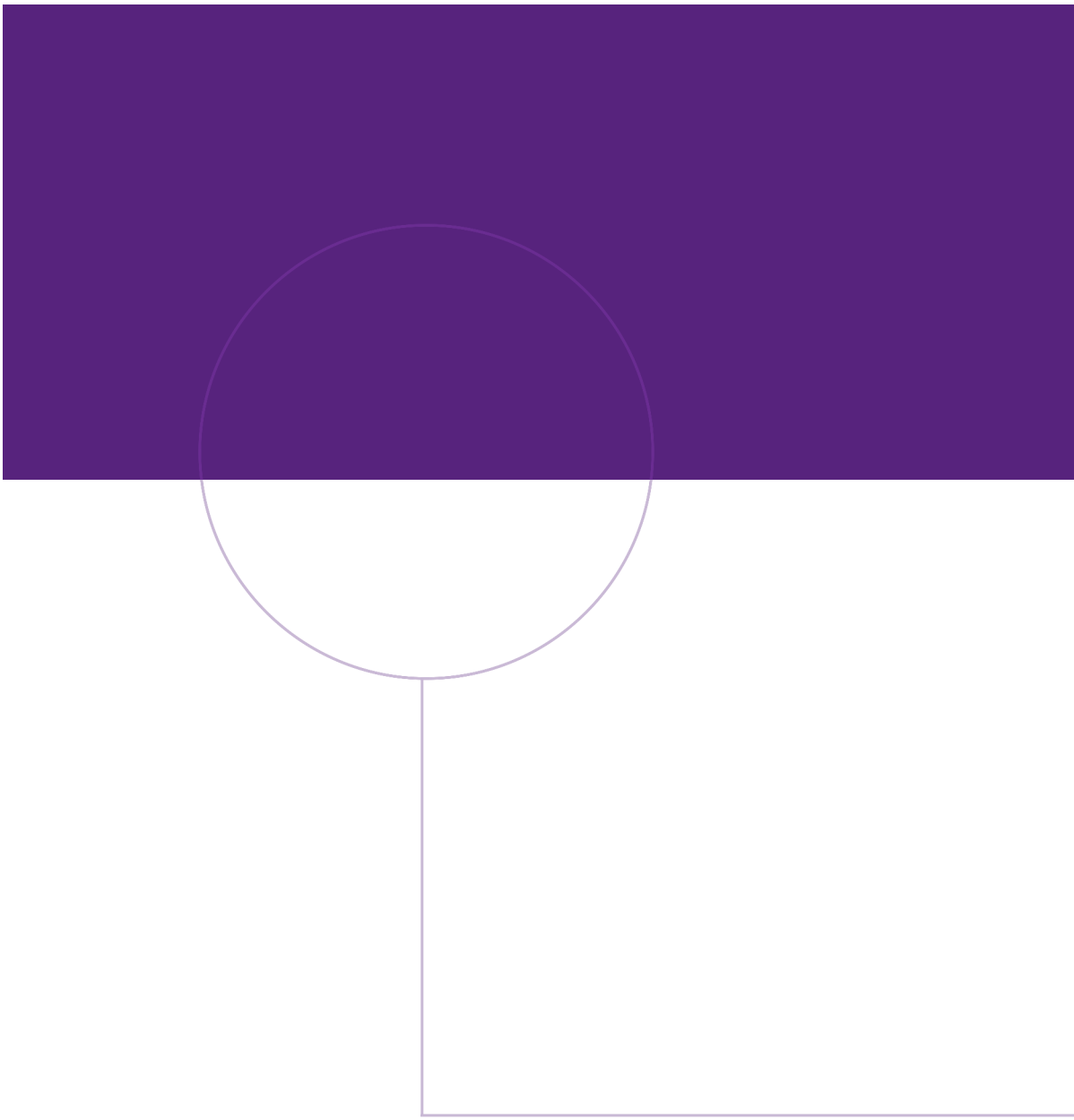


Figure D13: Neighbor-joining tree based on amino acid sequence, with Poisson method as a statistical method. Included bootstrap values to test for phylogeny, with 1000 replicates.

Appendix E

Table E4: Pictures of each specimen within the suggested species from phylogenetic analysis in Figure 7. Individual specimen numbers used during lab are also included for each specimen. Pictures of specimens 1-41 were taken one day after collection, while pictures of specimens 1B and 3B were taken more than one year after collection. Pictures: Marthe Svihus.

Haplotype group	Specimens						
1	2 	3 	4 	5 	6 	7 	8 
	9 	10 	11 	13 	14 	39 	40 
2	1B 						
3	28 	33 	36 	37 			
4	29 	31 	32 	34 	35 		
5	24 	25 	26 	27 	41 		
6	16 	18 					
7	1 	17 	19 	20 	38 		
8	21 						
9	12 						
10	22 	23 	3B				



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