SYSTEMATICS OF AFRICAN *VANILLA* ORCHIDS

LINKING MORPHOLOGICAL CLASSIFICATION AND DNA-BASED PHYLOGENY

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PREFACE

In this MSc Thesis, I looked into a small part of one of the genera from this large and intriguing orchid family: *Vanilla*. I applied both a 'classical morphological' as well as a DNA-based approach. These are quite different approaches and thus different research methods were needed. Therefore, both fields will be discussed separately with their own introduction, material and methods, results and discussion sections. Of course, I will also link both fields in a final discussion and conclusion.
Since this was quite a large project with many different aspects that did not always work out the way it was first anticipated, I chose to include all these aspects in this thesis. The thesis is thus outlined in chronological order including discussions about failed research methods and not built up in a strict scientific form.

Of course, I have had a lot of help during this project. First and foremost, I want to thank my supervisors Freek T. Bakker, Jan Wieringa, Theo Damen and Barbara Gravendeel for their guidance and constant support during this long-term project.
I especially want to thank Aline Nieman, Bertha Koopmanschap and Natasha Schidlo for all their help and advice in the lab; Kris van 't Klooster for his help on using Matlab; Marc Pignal and Ranee Prakash for their help in the herbaria of the Muséum National d'Histoire Naturelle in Paris and the Natural History Museum in London; the Alberta Mennega Stichting for making it possible for me to visit these herbaria; and of course all staff and students of the Wageningen Herbarium and the Biosystematics group!

SUMMARY

The goal of this MSc thesis is to link the classical morphological field of taxonomy to modern development of DNA based taxonomy for two small groups of Continental African *Vanilla* orchids: the *imperialis* and *africana* groups.

The taxonomy of species within the genus *Vanilla,* which is estimated to comprise 107 species, has long been an issue. Only in 2010, Soto Arenas and Cribb proposed a new classification describing two subgenera; the *Vanilla* and *Xanata*.
Vanilla has been used by humans for a long time. First by the Totonics from Southeastern Mexico and later by the Europeans after the Spanish colonisation.
All around the world, *Vanilla* species grow in the Pan-tropical region around the equator. Researchers do not agree how this distribution came about. Some state it is a result of radiation over Gondwanaland when it was not yet broken-up, other say long distance dispersal of seeds by for example bats could be an option.

Vanilla morphology is quite complicated with important characteristics found in the flower and leafs. The first part of this research concentrates on the classical morphological analysis of a number of these characteristics in the two groups of species. Principal Component Analysis (PCA) is used to search for trends. In the *imperialis* group, a number of recently newly described species are brought back to their original state. In the *africana* group, no final conclusions regarding the possible eight species can be drawn.

While the main part of morphological research on *Vanilla* dates back to the end of the nineteenth century, the DNA based research is only around fifteen years old. Here, I try to update the most recent cpDNA based phylogeny of Bouetard *et al.* (2010) in two ways. First using Ancient DNA methods developed in Leiden and second by using the more standard molecular systematic approached. The former method is used to incorporated species missing from the original phylogeny using material obtained from herbarium samples of the relevant species. For two of those, this method has been successful. The latter approach is used to incorporate sequences obtained from unidentified silica gel preserved fresh cuttings into the phylogeny. This has been successful for the *africana* and *imperialis* groups.

The flowers of Orchidaceae are important in species identification and are considered to be under selective pollinator pressure. No research has been done into the pollination of the species researched here, but using research in closely related species and genera some comments are given about this subject.

In the phylogeny of Bouetard *et al.* (2010), next to the *rbcL* region I used, also *psaB*, *psbB* and *psbC* have been used. These additional regions may be sequenced in future research using the material that is now available. In addition, the 5.8S nuclear ITS region might be interesting to research for the African species since a phylogeny is available comprising all American species.

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1. INTRODUCTION

1.1 ORCHIDACEAE

From as early as the seventeenth century, orchids were considered to form a family or order (Jarvis & Cribb, 2009; Tournefort, 1694). In his Éléments de Botanique (1694), Tournefort was one of the first to give a description of this family and in the first edition of the *Genera Plantarum* (1737), Linnaeus recognised eight different orchid genera containing 113 different species and varieties (Jarvis & Cribb, 2009). The then relative low number of described Asian and American orchids was a result of the Euro-centric origin of botany (Jarvis & Cribb, 2009). Now, the Orchidaceae are considered to comprise 925 genera containing about 27,135 orchid species ("The Plant List", 2010). Orchidaceae are widely distributed around the world with multiple genera and species in almost every part in the world except for large parts of the arctic regions.

Being one of the largest families in the angiosperms (Dressler & Dodson, 1960; Heywood, 1993) and one of the most complex flowering plant families (Cameron, 2004), there has been a lot of speculation about the age of this family. Since the fossil record of Orchidaceae is low, it has for a long time been difficult to accurately date the family. Early researchers stated that the family was of a recent age because of the complicated floral morphology and specialized pollination process. On the other hand, the fact that orchids are found all around the world would suggest to other researchers an ancient origin in times of the Gondwana supercontinent (Ramírez, Gravendeel, Singer, Marshall, & Pierce, 2007). In this continental-drift hypothesis, describing the radiation of the family around the world as a result of the break-up of Gondwanaland, it is suggested that the family is as old as 188–455 My. Other hypotheses estimate the crown radiation of the family to be between 68–104 Mya (Bouetard et al., 2010). However, with the help of a newly found fossil it has revealed that the origin of the family probably lies between 76-84 Mya (Ramírez et al., 2007).

It has been difficult to form a satisfactory classification for this family because the flower, one of the most important characteristics used to identify species morphologically, is thought to be under selective pollinator pressure and is thus subjected to much morphological change (Bouetard et al., 2010; Cameron, 2004). Nevertheless, over the years many, both classical (Dressler & Dodson, 1960; van der Pijl & Dodson, 1966) as well as DNA based (Cameron, 2004; Cameron et al., 1999; Freudenstein et al., 2004) revisions, monographs and articles about Orchidaceae taxonomy have been published. Still, orchid taxonomy remains complicated and subjected to constant improvement.

1.2 THE GENUS *VANILLA*

1.2.1 VANILLA TAXONOMY

The placement of the *Vanilla* genus amongst sister genera in the family has been a long standing question. However, with the development of DNA-based comparisons, a lot of the uncertainties have been cleared away and the position of *Vanilla* within the family has been defined. Within the Orchidaceae, five subfamilies are now recognised, one of which is called Vanilloideae (vanilloid orchids). This subfamily comprises two tribes, the Pogonieae and the Vanilleae, of which the latter is considered to encompass about fourteen genera. It is in this tribe that the genus *Vanilla* is now situated (Figure 1) (Séverine Bory, Grisoni, Duval, & Besse, 2007; Bouetard et al., 2010; Cameron, 2003, 2011b)

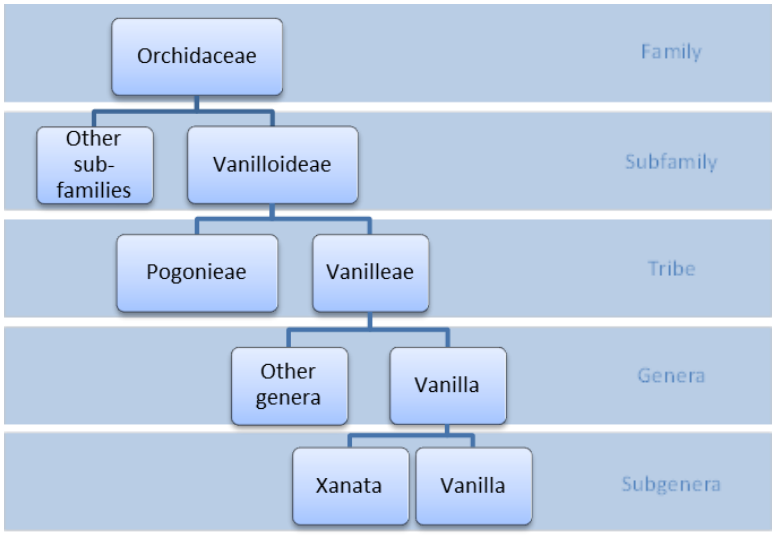

FIGURE 1. PLACEMENT OF THE GENUS VANILLA IN THE ORCHIDACEAE.

Many new species have been described over the past 120 years while an objective classification of the genus was lacking. The first to compile a morphology based classification was R.A. Rolfe in 1895. He divided the genus in the sections *Foliosae* and *Aphyllae*, with and without having fully developed leaves subsequently. At that time, there were about 51 *Vanilla* species known (Rolfe, 1895). Then, for the next 50 years no revising work on *Vanilla* was published. In 1954, Portères was the next to compile all the new *Vanilla* species in an updated classification. His work was published in 'Le Vanille et la Vanille dans le Monde' edited by Bouriquet (1954). In this work, leading researchers published chapters about their knowledge on *Vanilla*, ranging from morphology and anatomy to taxonomy and from ecology to chemistry.

Portères accepted the earlier classification of Rolfe, but refined it by naming three sub-sections within the section *Foliosae*: the *Papilloseae*, *Lamelloseae* and *Membranaceae* (Portères, 1954). These subsections differ in thickness of the leaves (*Papilloseae* and *Lamelloseae* vs *Membranaceae*) and appearance of the lamellae (*Papilloseae* vs *Lamelloseae*). This classification was widely accepted by *Vanilla* researchers, until in 2003 Soto Arenas stated the name of the section *Foliosae* was not valid since according to the Botanical Code of Nomenclature (McNeill et al., 2006) a proposed subdivision of a genus which contains the type species of the genus must repeat the genus name. Also, no Latin descriptions were given and no type specimen was appointed for the new subsections by Portères, therefore they are nomenclaturally invalid (Soto Arenas, 2003). However, the classification of Portères remained accepted until 2010, when Soto Arenas & Cribb proposed a completely new infrageneric classification. The old sections and subsections were removed and instead two new subgenera were described: *Vanilla* and *Xanata* (Figure 1). The latter is divided in two sections: *Xanata* and *Tethya*. Combined, the two subgenera are estimated comprise some 107 species of *Vanilla* distributed around the world (Miguel A. Soto Arenas & Cribb, 2010).

1.2.2 ETHNOBOTANIC HISTORY

The history of the use and cultivation of *Vanilla* by humans is a long one. There are multiple legends about the origin of *Vanilla* known from the Totonics, indigenous to the southeast Mexican region Papantla, the modern Vera Cruz (Cameron, 2011a; Correll, 1953; Ecott, 2004; Pesach Lubinsky, Romero-gonz, Heredia, & Zabel, 2011). According to one of these legends, the *Vanilla* orchid arose from the spilled blood of a beautiful Totonic prinsess (Cameron, 2011a; Ecott, 2004).

The Totonics are said to be the first people to have used *Vanilla* in everyday life. Not as a flavouring as we know it now, but to perfume their houses (Ecott, 2004). When the Totonics were suppressed by the Aztecs, vanilla was used to pay tribute to the Aztec emperors. From archaeological remains it is known that the Aztec elite used to drink xocoatl, a thick chocolate drink flavoured with honey, peppers, maize and vanilla. Supposedly, Hernando Cortés was the first white man to taste vanilla flavoured chocolate when meeting the Aztec emperor Montezuma in 1519 (Correll, 1953; Ecott, 2004; Pesach Lubinsky, Bory, Hernández, Kim, & Gómez-Pompa, 2008). The Spanish were impressed with the regenerating ability of the potent drink, saying that 'a cup of this precious drink enables a man to walk for a whole day without food' (Ecott, 2004). After they were finally able to unravel the mystery of the origin of vanilla, the Aztecs were guarding their secret jealously, the import of vanilla from Mexico to Spain was started.

For many years the Spanish had the monopoly on the vanilla trade, since the plant would not produce fruits when it was first cultivated in the 1730's and pods were thus only produced in the Spanish colonies. There was, however, a lot of interest from both England and France to break the Spanish monopoly (Cameron, 2011a). The problem was that when the *Vanilla* species most suitable for cultivation, the original Mexican *V. planifolia,* was imported to Réunion and Madagascar for cultivation, the climate was right but there was no natural pollinator. Eventually, the French discovered that the fruits would set when the flowers were pollinated by hand and the Spanish monopoly was broken (Cameron, 2011a; Ecott, 2004). This was a time consuming and non-effective method. It was not until the early nineteenth century that the slave Edmond Albius discovered a relative easy way to fertilize *Vanilla* flowers (

Figure 2). Although he was never really rewarded for his discovery, Albius's method is now widely used in *Vanilla* cultivation and nowadays, all the fruits imported from Madagascar and Réunion have been fertilized in this way (Cameron, 2011a; Ecott, 2004).

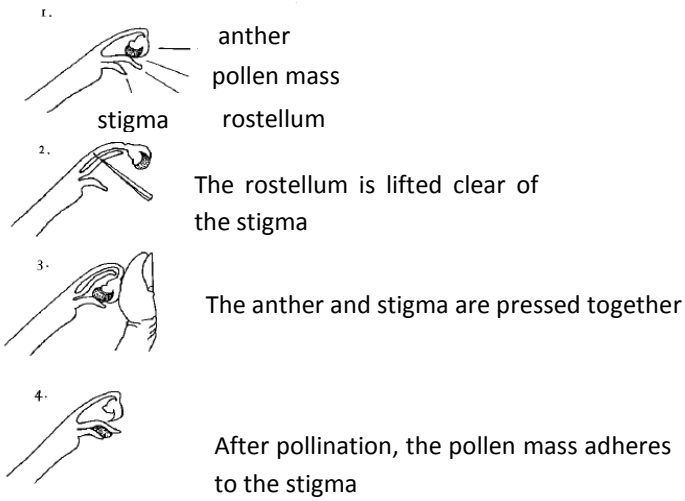

FIGURE 2. METHOD FOR HAND-POLLINATION DEVELOPED BY ALBIUS (AFTER ECOTT, 2004).

Vanilla is still one of the most desired products in both the food and perfume industries. There are three species of *Vanilla* that are commercially suitable since they produce the typical vanilla fragrance in high, yet different, quantities. These are the 'original' *Vanilla planifolia* from Mexico*, V. pompona* and *Vanilla tahitensis* (Stern & Judd, 1999). However, there appear to be many more aromatic species (Pesach Lubinsky et al., 2008).

The farmers that grow *Vanilla* sell their fruits to a curer when they have been growing on the plant for around nine months. This curer is the one who converts the thick, green fruits into the slim, oily, brown pods we buy in the supermarket (Bruman, 1948; Cameron, 2011a; Ecott, 2004). The curing of a vanilla bean is a long and difficult process. The green fruits have to be boiled first to stop the natural ripening process. Then, the beans have to be sweated for the vanillin, responsible for the vanilla flavour, to develop during dehydration of the fruit. Too quick or slow dehydration will result in either a too dry or a musty bean ruining the crop. After the sweating, the pods are dried by sun and wind. In all the places where vanilla is produced as a cash crop the main stages of curing, sweating and drying are the same, but there are differences in the execution (Cameron, 2011a; Ecott, 2004).

1.2.3 *VANILLA* BIOGEOGRAPHIC DISTRIBUTION

When looking at the global distribution of the vanilloid orchids and *Vanilla* in particular, the transoceanic distribution is striking (**Error! Reference source not found.**). This genus occurs on all continents, except for Australia and the Arctic regions. There seem to be two hypotheses explaining this wide distribution. The first suggests that the shape of the fruits of the *Vanilla* orchid makes long-distance dispersal by for instance birds and bats possible (Cameron, 2011a). The other possibility is that *Vanilla* radiated over Gondwana before the breakup into continents (Bory et al., 2007; Bouetard et al., 2010; Cameron, 2011a; Dressler, 1981; Ramírez et al., 2007).

There is unfortunately no consensus on a general historical biogeographic hypothesis for *Vanilla*. As described above, researchers date the crown radiation of the Orchidaceae between 76-84 My old. Based on this estimation, some say that the pantropical distribution proposed by Dressler (1981) is possible and the genus *Vanilla* could have radiated around Gondwanaland before the continents were too far apart (Pesach Lubinsky et al., 2008; Ramírez et al., 2007). However, as Bouetard *et al.* (2010) point out, that would be inconsistent with the age of the *Vanilla* clade inferred when determining the age of the *Orchidaceae* clade: 34,49 My. At that time in the Eocene, the landmasses are considered to have already separated. Since there also seems to have been a second dispersal event radiating from Africa back to the Caribbean, a long distance dispersal of *Vanilla* seems a possible explanation (Bouetard et al., 2010). However,

FIGURE 3. PAN-TROPICAL DISTRIBUTION OF THE GENUS VANILLA AROUND THE WORLD (AFTER DRESSLER,1981).

the radiation back to the Caribbean as described by Bouetard *et al.* (2010), is not certain, since the phylogenetic inference doesn't include possible sister species of the species that dispersed back from Africa.

2. RESEARCH QUESTIONS & HYPOTHESES

Historically, *Vanilla* taxonomy was always based on morphological characteristics. Even now, when molecular systematics and phylogenetic analysis is so far developed, no complete consensus classification of the *Vanilla* orchids has been formed linking these two taxonomical fields.

The aim of this study was to link these two because they are both important in modern taxonomy. Since the subjects are different, the research questions are divided over the two fields to give them both the attention they deserve. An introduction into the fields and accessory literature whereupon I based these questions and hypotheses is provided in the following chapters. In Chapter 6 I will discuss the pollination biology of *Vanilla* and in Chapter 7, I bring these fields together in a final discussion.

2.1 MORPHOLOGICAL CLASSIFICATION

1. Is the species named *V. ochyrae* Szlach&Olsz. (1998) in fact *V. imperialis* Kraenzl.?

Recently, T.H.J. Damen from Naturalis Biodiversity Center found that there is some inconsistency in the description of *V. imperialis*. Although all the authors refer to the work of Rolfe being the first to provide an infrageneric classification of the *Vanilla* genus, none of them take into account the typification of *V. imperialis* Kraenzl. by Kraenzlin (Kraenzlin, 1896). The type specimen (*Zenker & Staudt. 626*) appears to be lost when the Berlin Herbarium was destroyed during bombing of the city in 1943, but in the publication a clear drawing of *V. imperialis* was provided. Also, the German text accompanying the Latin description clearly states that 'die Lippe selbst ist nur vorn frei und bildet dort eine queroblonge Platte' meaning that the lip (of the flower) itself is only free at the front and forms there a square-like plate. In the later monograph of Portères (1954) the description of *V. imperialis* seems to be comparable with the one of Kraenzlin, especially since he used the drawings from Kraenzlin in his own description. However, in the Flore du Cameroun of Szlachetko & Olszewski (Szlachetko & Olszewski, 1998), the description and drawing of *V. imperialis* do not agree with those of Kraenzlin (1896) but the drawing of the newly named *V. ochyrae* Slzach&Olsz. is similar to the one of Kraenzlin and at first sight no clear difference can be found. Also, in the revision of Soto Arenas & Cribb (2010), the only difference between *V. imperialis* and *ochyrae* is the shape of the plate of the lip, having either a triangular or an elliptic to semi-circular apex. Clearly, there is a problem with the descriptions of these species.

Considering the discrepancy in the descriptions of *V. imperialis* arisen through the years I hypothesise that:

a. *V. imperialis* described by Kraenzlin (1896) was mixed up by Szlachetko and Olszewski with another *Vanilla* species and the new name *V. ochyrae* Szlach&Olsz. was given to the original *V. imperialis* Kraenzl.

b. *V. imperialis* sensu Szlachetko & Olszewski (1998) is conspecific with the type of *V. lujae* De Wild.

2. How many (sub)species are present in the *V. africana* group (Miguel A. Soto Arenas & Cribb, 2010) (based on an analysis of a number of morphological characteristics)?

In the informal *V. africana* group, eight species (*V. crenulata, africana, acuminata, cucullata, hallei, heterolopha, ramosa* and *zanzibarica*) have been recognized by Soto Arenas and Cribb (2010). However, according to the World Checklist of Monocotyledons (Govaerts & Campacci, n.d.) *V. crenulata* is not accepted by Geerinck (Geerinck, 2011) who place it in synonym to *V. africana*.
In addition, the species *V. ramosa* has in the past been treated as synonymous to *V. crenulata* by Geerinck, which only adds to the confusion (Geerinck, 2011; Miguel A. Soto Arenas & Cribb, 2010). In addition, both *V. ramosa* and *cucullata* are recognised as subspecies of *V. africana* in the Flore du Cameroun (Szlachetko & Olszewski, 1998). These subspecies were later restored to species level and are now considered valid species based on differences in lip characteristics. But Soto Arenas & Cribb are not confident that *V. cucullata* is not synonym to *V. crenulata* (Miguel A. Soto Arenas & Cribb, 2010; "The Plant List," 2010).

Because of this disorder found in the *V. africana* group both in the past and in the present**,** I hypothesise that:

a. The entities named *V. crenulata, africana, acuminata, cucullata, hallei, heterolopha, ramosa* and *zanzibarica* as described by Soto Arenas & Cribb (2010) are indeed separate species.

2.2 DNA-BASED PHYLOGENETIC INFERENCE

3. Can (part of) the phylogenetic hypothesis of Bouetard *et al.* (2010) be complemented and optimised with additional *Vanilla* species, especially those from the *V. imperialis* & *africana* groups as described by Soto Arenas & Cribb (2010)?

When comparing the phylogenetic tree based on cpDNA of Bouetard *et al.* (2010) to the infrageneric classification of Soto Arenas & Cribb (2010) it can clearly be seen that the informal groups of the classification are comparable to the clades formed in that phylogeny (Figure 4). The *V. africana* and *imperialis* groups, which are of interest in the previous research questions, are arranged along the phylogenetic tree and are well supported by the Bayesian node support probabilities. The species of the other informal groups can also be arranged along the phylogenetic tree and form clear uniform groups.

The phylogenetic tree of Bouetard *et al.* (2010), however, is far from complete. Many species are missing from the sampling, especially in the Old World & Caribbean clade. Of most informal groups only a few representatives are included while the remaining species are missing (e.g. *V. acuminate, cucullata, hallei, heterolopha, ramosa, zanzibarica, grandifolia* and *ochyrae*), and two informal groups (*V. francoisii* and *chalotii*) are completely missing from the phylogenetic tree. In addition, many sequences used in the analysis seem not yet to have been properly identified to species level since for many lineages only the accession numbers in GenBank are provided. Also, it is interesting that the two sequences of *V. africana* that were used in the analysis do not group together but group in two unrelated positions, with one of the two more closely linked to *V. crenulata* than to its sister sequence. Last, the line colonizing the Caribbean is quite intriguing since it appears to be originated from an African line that is separate from the other African lines and there is no taxonomic identification available for this particular sequenced African accession.

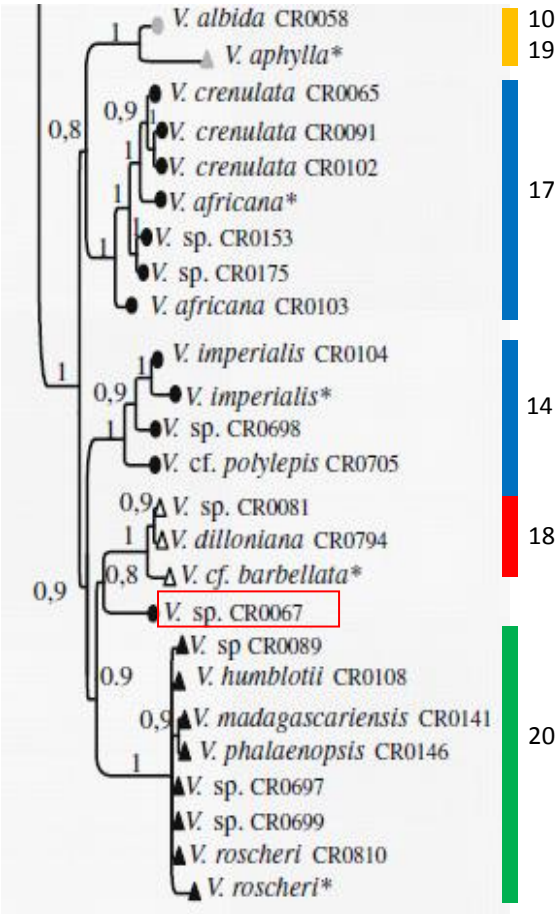

FIGURE 4 PHYLOGENETIC TREE OF BOUETARD ET AL. (2010) WITH THE INFORMAL GROUP NUMBERS OF SOTO ARENAS & CRIBB (2010)(10: V. ALBIDA GROUP, 14: V. IMPERIALIS GROUP, 17: V. AFRICANA GROUP, 18: V. BARBELLATA GROUP, 19: V. APHYLLA GROUP, 20: V. PHALEANOPSIS GROUP). COLOUR INDICATES GEOGRAPHICAL REGION: ASIA (YELLOW), CARIBBEAN (RED), CONTINENTAL AFRICA (BLUE) AND MADAGASCAR (GREEN) (AFTER BOUETARD *ET AL.* (2010).

Therefore, I proposed to complement and optimise the phylogenetic tree of Bouetard *et al.* (2010) using sequences of the additional African *Vanilla* species as described in the classification of Soto Arenas & Cribb (2010).

Based on the problems with the phylogenetic hypothesis as described above, this phylogeny can be complemented by adding additional species from the already analysed clades and implementing lacking clades. The associated hypotheses are:

a. The *V. chalotii* group will cluster in an intermediate clade between African and Caribbean species.

b. The *V. francoisii* group will be closely related to the *V. madagascariensis* group, since both are Indian Ocean based groups.

c. The informal *V. imperialis* and *africana* groups are monophyletic groups.

3. SPECIES CONCEPTS

Before diving into the actual research and forming conclusions whether entities are species or not, I think it is important to take a closer look into the question 'What is a species'. Unfortunately, for many researchers this is one of the most difficult questions one can be asked. The concept 'species' is one of the most fundamental concepts in biology, but (as is often said) there are as many opinions about what a species is as there are biologists. For every field in biology, a different set of aspects that together make up the concept species is relevant. This is why it so hard to form a general consensus, since the interests are often quite contradictory.

In a lot of the formulated concepts, all kind of processes as evolution, speciation and mutation are taken into consideration. But is that necessary? Does it matter to make all these processes, difficult in itself, part of your species concept?
The question is then whether it is your goal to form a definition of a species in the light of these evolutionary processes, or is it your goal to distinguish and describe entities we assign the rank of species? I think there is quite a difference in these mind sets and that doesn't help. Some researchers are only interested in accurately describing species as groups we can find in nature. Their goal is to describe on what morphological grounds one group is different from another. Solely based on what they see in the present state of the plants. That is quite a difference from other researchers who are not interested in describing a particular species on its looks, but are interested in for example differences in behaviour and use species in their research (Hausdorf, 2011).
But is not the goal of a general used species concept also to describe what Wilkins calls *logical* species, so including evolutionary processes, and not 'just' to define *biological* species 'which are diagnosed by morphological characteristics'?

Ultimately, every researcher has to formulate his or her own species concept. At the moment, around 26 heavily discussed species concepts are in circulation and everybody chooses certain parts of them that they can use to form their own working concept (Frankham et al., 2012; Wilkins, 2003).
Personally, I find the species concept quite a difficult problem. I have never given much thought about the species concept question before. I find it thus difficult, with my lack of knowledge of the plant phylum, to form a solid species concept. How can one base an underpinned opinion about such an important concept on so little knowledge? Also, there are numerous examples of researchers changing their opinion on the species concept as their years in research advance and their personal experience increases.

Before I started this thesis, I was quite happy with the old fashioned taxonomic practise to differentiate species based on a number of correlated morphological characteristics the individuals within the species have in common but clearly separate them from other individuals. This method is based on the Morphological species concept that states that a population should be morphologically distinct from another population to make them separate species. Usually, a number of three or more distinct characteristics is found sufficient to make this separation. Subspecies or varieties can be recognised in much the same way. When less than three correlated morphological characteristics can be found to separate the one entity from another but there is a clear separation in geographical or ecological sense, they may be called subspecies. If no such additional separation can be found but the morphology still implies a difference, the term variety is used (Sosef, personal communication). It is quite practical to define a species in this way when performing morphological work. But the number three is quite arbitrary. And in the USA the rules for subspecies and variety used to be the other way around. And as Wilkins writes: '... living species were always understood to include or require a *generative power* rather than morphological similarity or identity, which was always held to be a way of identifying them at best.'
The outlines of this morphological practise are thus no longer enough for me. In the end, a good species concept should define the underlying processes, but should also be fit to use in real life.

As I said earlier, there are about 26 species concepts. But it is difficult to adopt an already existing species concept when every concept has its pitfalls, is vague or not complete. From my gut feeling, I would say that the Biological species concept ('Species are reproductively isolated units in that, by definition, only conspecific matings yield fertile offspring', Zachos & Lovari, 2013) makes sense. If two entities can form fertile offspring, how can they be so different that they can be called separate species? But in plants, it is known that fertilisation can occur even over genus boarders (Wilkins, 2003; Zachos & Lovari, 2013). Also in my clade, species are found to hybridise. Either by accident (*V. claviculata x barbellata*, Nielsen, 2000), or because they are made to (*V. tahitensis,* P. Lubinsky et al., 2008).

In the genetic sense, I can relate to the concept of Templeton: A species is '[t]he most inclusive group of organisms having the potential for genetic and/or demographic exchangeability (Wilkins, 2003). However, as Wilkins points out, this is more of a way to describe the underlying speciation processes and not useful for identifying species (Wilkins, 2009).

Also, when one is taking the genetics into consideration, should you not also take in the evolutionary time process? Here, the characterisation by E.O. Wiley 'A species is a single lineage of ancestral descendant populations of organisms which maintains its identity from other such lineages and which has its own evolutionary tendencies and historical fate' makes sense. In this light, some people say that once a species has evolved, it remains stable (Wilkins, 2009). But is this the case? When you perform a lab experiment with a bacterial strain of some species and you let it evolve under a certain set of environmental conditions, did you then create a new species? Or is it still the same, but adapted to the new environment?
And again where do you draw the line? Any new species descends from another already existing species. So all organisms that form the new species are descendants from multiple organisms from the mother species. In the light of this concept there thus should only be one species, the one all life originated from.

With the Phylogenetic species concept we seem to circle back to the concept we started with. In this concept, species are defined by their autapomorphies, their derived characteristics. Species have their own set of autapomorphies that makes them unlike other species in the same genus, but they share the same synapomorphies. These are unique on the higher taxonomic genus level. In its original form the Phylogenetic species concepts states that 'A species is the smallest diagnosable cluster of individual organisms within which there is a parental pattern of ancestry and descent (Wilkins, 2009; Zachos & Lovari, 2013).
In some versions, the aspect of monophyly is added (a monophyletic group being an ancestral species and all its descendants). If species ought to be monophyletic, they can be defined by their apomorphies, their derived characteristics. However, under this assumption, a parent species gives rise to two daughter species and then ceases to exist. I don't necessarily think that to be the case. It could be that a group of individuals of a species splits of and evolved to become a species different from the one they originally were. But that doesn't mean the old species is gone per se.
Again, I can find myself in this concept. It seems quite logical and usable in practise. You can statistically test whether there is overlap in morphology or something else and when there isn't, you have more than one species. But as Zachos & Lovari, (2013) point out, 'biological reality has been sacrificed on the altar of testability'. Because where do you draw the line? Any two individuals may look the same, but with molecular techniques every individual is different based on the mutations that may have occurred. '... every and any population of every and any species will contain dozens or hundreds of diagnosable units or, under the diagnosability PSC logic: species...'(Zachos & Lovari, 2013). Under the same argument, the monophyly part also doesn't hold up (Zachos & Lovari, 2013).

In my musings, I found all of these aspects to be important enough to be part of my species concept. So a species should be reproductively isolated (while allowing for hybridisation), has the potential to exchange its genes with other organisms in its group, has an ancestral component and is defined by a set of exclusive autapomorphies.

Still, this only gives a general outline of what a species should represent. I yet have to come to a hands on definition that I can easily use in my day to day research.

4. MORPHOLOGICAL CLASSIFICATION

In this chapter, I address the first two research questions:

1. Is the species named *V. ochyrae* Szlach&Olsz. (1998) in fact *V. imperialis* Kraenzl.?
2. How many (sub)species are present in the *V. africana* group (Miguel A. Soto Arenas & Cribb, 2010)

Therefore, I first give an introduction into the morphology of *Vanilla*.

4.1 INTRODUCTION

The Vanilloideae is the only subfamily in the Orchidaceae that contains genera with climbing plants and their habit is completely adjusted to this lifestyle. When recently germinated, the plants are terrestrial and grow on the forest floor. In this stage, the roots penetrate the soil layer and take in the nutrients. When developing, the plant starts to climb into a tree and aerial roots are formed at each leave-node. At this stage, the plant has become a vine. When the basal part that connects the plant to the soil dies off, the plant has become an epiphyte. The roots are used to stabilize the stem on the tree trunk and take up nutrients from fallen leaves lying on the trunk and water from the humid air and rolling down the tree when it has been raining. Over the years, the vine grows up the trunk into the canopy where it can flower (Cameron, 2011a; Fouché & Jouve, 1999; M. A. Soto Arenas & Cameron, 2003).
It is difficult to determine whether the *Vanilla's* are truly terrestrial or epiphytic because of their changing lifestyle. Therefore, they are often said to be hemi-epiphytic, being a bit of both (Cameron, 2011a).

From the start of *Vanilla* classification, flower characteristics have always been important for the identification of the different species. These flowers grow in such a way that the chance of fertilization when visited by a pollinator is fairly large. This is important, since the flowers open early in the morning just before dawn and wither before noon. The chance of a pollinator being present at just the right time, and carrying suitable pollen, is therefore only small. To increase the reproductive chances, *Vanilla* vines are able to produce quite large quantities of flowers that do not flower all at the same time (Cameron, 2011a; Ecott, 2004; Fouché & Jouve, 1999).

To facilitate the visitation of a pollinator, the flower is twisted 180° and the labellum forms a landing platform for the pollinator. Also, the labellum is fused with the other petals and forms a tube. The bee or other pollinator is drawn to the flower by the promise of nectar. It lands on the labellum and enters the tube easily. However, when it tries to leave the tube, it is hindered by a callus consisting of hairs or scales. These features lay flat when the pollinator enters the flower but stand up when it tries to leave, thus preventing an easy retreat. The pollinator has to wiggle backwards and climb over the callus. In doing so, it has to lift its abdomen and then touches the anther holding the pollen. Since the pollen is nicely packaged together in a clump, it sticks together and to the abdomen of the pollinator. When visiting the next flower, the pollen is delivered to the stigma when the pollinator enters the flower (Cameron, 2011a; Ecott, 2004)11) (Figure 5).

Since the anther and stigma are fused together and form the column, there is a fair change of self-fertilization. To prevent this, a little flap called the rostellum has developed to separate the two. It is this flap that has to be circumvented when pollinating the flowers by hand (Severine Bory et al., 2008; Cameron, 2011a) (Figure 2 and 5).

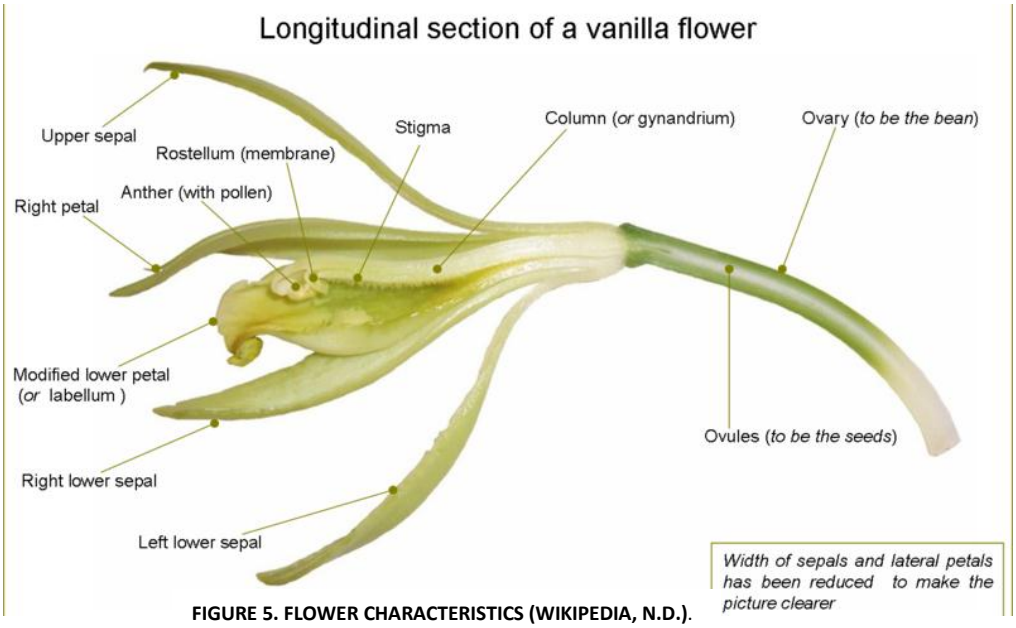

FIGURE 5. FLOWER CHARACTERISTICS (WIKIPEDIA, N.D.).

The leaves of the *Vanilla* vines are also of importance when identifying the species. Some species clearly have only rather thin, membrane like leaves or seem to lack them completely. When plants do have leaves, they can differ greatly in shape, size and colour. Even in one plant, the leaf morphology cannot be said to be constant. The only species clearly distinct from all the other *Vanilla's* is *Vanilla grandifolia*, which is the only one with strikingly round leaves compared to the usual elliptic shape (Cameron, 2011a; Stern & Judd, 1999).

4.2 Material and Methods

4.2.1 Morphology

Extensive morphological analyses had to be performed to gather the data needed to answer the first two research questions.

The first step in this process was to request herbarium specimens of *V. ochyrae, imperialis, polylepis, grandifolia, crenulata, africana, acuminata, cucullata, hallei, heterolopha, ramosa* and *zanzibarica* on loan from the herbaria of Kew Royal Botanical Gardens (K), Paris (P), Brussels (BR) and the British Museum (BM), New York (NY) and Madrid (MA).

V. ochyrae, V. lujae and *imperialis* needed to be compared to answer the first research question. Together with *V. polylepis* and *grandifolia*, they form a monophyletic group in the phylogenetic tree of Bouetard et al. (2010) and one informal group in the revision of Soto Arenas & Cribb (2010). The same is the case with the other species that together form the *africana* group.

Because it is desirable to study as many specimens of these species as possible in this kind of research, I visited Kew Royal Botanical Gardens, the National History Museum in London and the Muséum National d'Histoire Naturelle in Paris. There, I selected suitable specimens for the loan while I measured the other specimens on site. Appendix 1 gives an overview of all available specimens.

All measurements were performed on boiled flowers with a ruler. Measurements were entered in an Excel database for further use in the statistical analyses (4.2.2 Statistical Analysis). All specimens were photographed for future use as elaborate and detailed as possible. Good flower specimens were kept on alcohol during the research period.

In addition, all data available from the labels on the specimens was entered in the Brahms database. The Meise herbarium of Brussels sent over their databases, which were incorporated in the Brahms database. Then, Theo Damen searched appropriate coordinates for all mentioned locations. Using Arcview I then made appropriate distribution maps of all relevant records.

4.2.1.1 Imperialis group

In the case of the *imperialis/ochyrae* combination, I took a close look into the shape of the floral lip since it is a characteristic that clearly distinguishes *V. imperialis* from *V. ochyrae* as described in the Flore du Cameroun (Szlachetko & Olszewski, 1998). The first (sensu Szlachetko & Olszewski) having a pointed lip apex and the latter having a more quadrangular shaped lip apex. However, when looking at the drawing available from the original protologue of *V. imperialis* by Kraenzlin, it became clear that the flower there also has a quadrangular shaped lip apex. This is comparable to the shape of the lip of *V. ochyra*e of Szlachetko & Olszewski (1998) and not to the drawing there available *of V. imperialis*, which is much more pointed. Therefore, I looked into the possibility of a gradient in lip pointedness within the *imperialis/ochyrae* combination.

I calculated this pointiness by measuring the angle between the central line along the lip and the farthest point of the lip (Figure 6). Both sides of the lip where measured in this way when possible.

I also looked into other characteristics that might be used to distinguish the two species other than lip apex shape. I looked into the way the rest of the flower is attached to the column and measured the length and width of the lip and the column. In addition, I looked at the hairs on the lip, since in the literature this is used to help distinguish the species from one another. I also looked at non-floral characteristics as the bracts (their shape and how they are attached to the inflorescence) and the leaves.

Next to this morphological study, I also studied the available literature that is mentioned above in more detail.

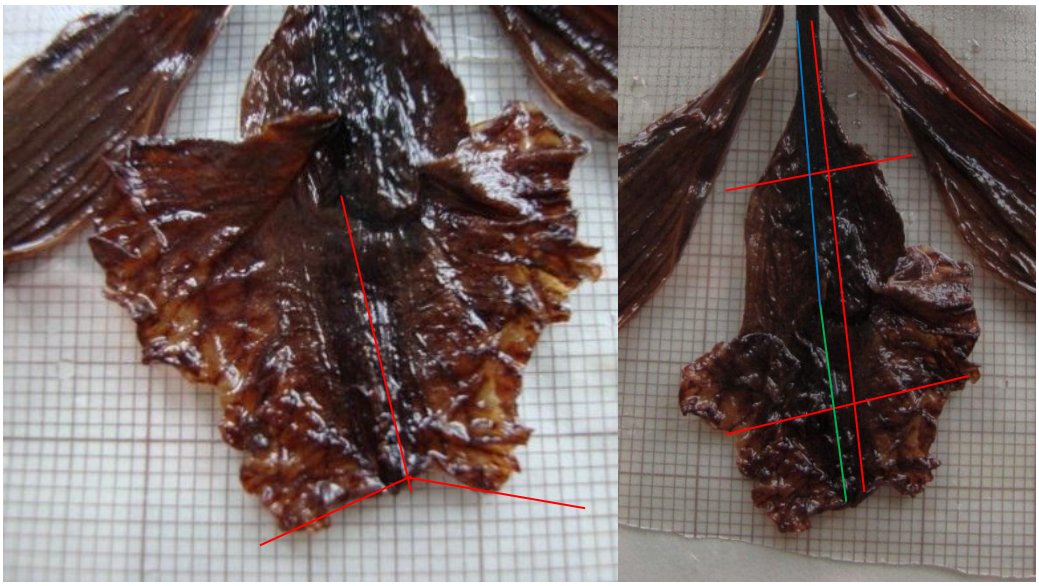

FIGURE 6. MEASUREMENTS ON *V. IMPERIALIS*. LEFT: MEASUREMENTS TAKEN FOR POSSIBLE LIP GRADIENT. RIGHT: ADDITIONAL MEASUREMENTS.

4.2.1.2 AFRICANA GROUP

In the case of the *africana* group, the group of species that is part of the second research question, I looked at another set of characteristics since the flowers are quite different. Beforehand I performed no literature research into the characteristics distinguishing the entities in this group. In this way, I could remain objective about which characters were to be measured since I did not know which were supposed to be of interest for each species.

To ensure this objectivity, I started with sorting all the available specimens by collector and number instead of identification. This helped with spotting the differences among the overall specimens and the flowers.
Then I looked at the flowers under a binocular and took photographs of everything I thought could be interesting or relevant. The photographs were not only to keep record of interesting characteristics, but also for use in the program tpsDig2 (Rohlf, 2010). This morphometric program can automatically calculate distances from a set combination of points that together define the characteristics studied in an organism, in this case the flower. First I converted the JPG-files of the pictures to .TPS-files, the extension used by tpsDig2, with the utility program tpsUtil (Rohlf, n.d.). Then the files could be loaded in tpsDig2. After a trial run however, I found this method was not suitable for this type of flowers (see 'Discussion and Conclusion' section in this chapter for further explanation). Therefore further measurements were taken by hand.

Error! Reference source not found.shows all the measurements I performed on the flowers of this group. To begin with, I measured the entire length of the column and lip (distance between points 1-2 and 3-2). Then, I measured the length of the lip that was visible (not hidden behind the side of the flower tube, points 1-4) and the length from the lip apex till the fans (points 1-5). I also measured the angle between the column and lip (angle at point 2) and the angle of the two curves in the column (points 6 and 7). When present, I also measured the length and height of the augmentation on the lip. Also, I counted the number of fans on the callus when possible.

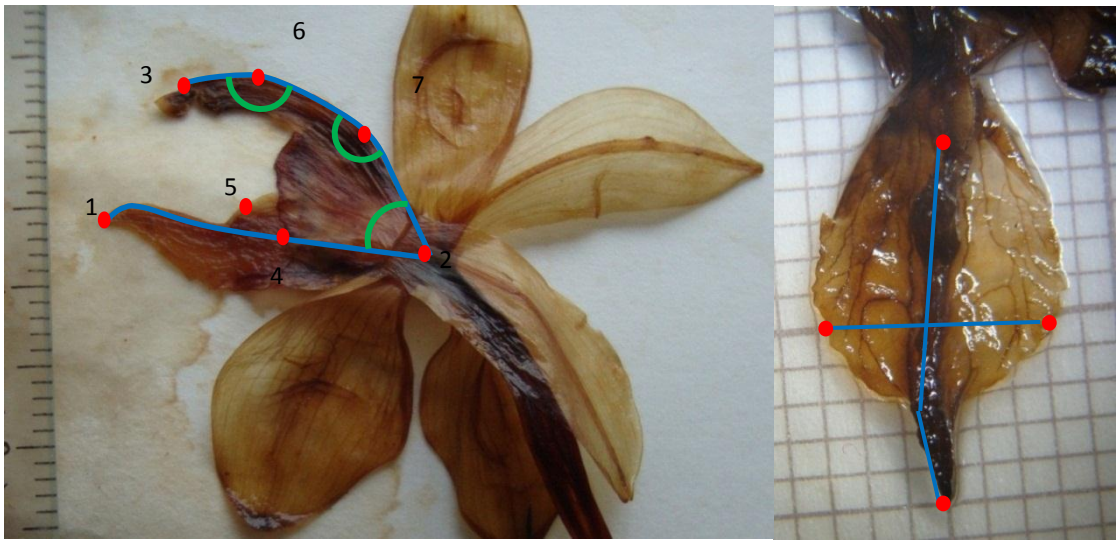

FIGURE 7. EXAMPLE OF THE MEASURED FLORAL CHARACTERISTICS ON J.C. BOWLING, GC38137 (LEFT), AND J.O. AREWAODO, JOA.648 (RIGHT).

Since many living *Vanilla* specimens growing in greenhouses around the world never flower because it is so difficult to provide the right conditions, it would be a great help if we would be able to distinguish the different species based on other characteristics than those present in the flowers.
To find out whether there are any usable vegetative characteristics, I first divided all the available specimens over different piles based on the shape of their leaves 'by eye' and recorded these groups.

It is quite rare and difficult to obtain a wild, flowering *Vanilla* orchid. However, it is a bit more common that an inflorescence is present. In a subset of specimens that turned out to be of relevance in flower distinction (see Results section of this chapter), I searched for additional characteristics for identification of the species in this way. Theretofore, I looked at the peduncle and form of the inflorescence itself.
No measurements were taken in this case, but different piles were formed 'by eye'.

4.2.2 Statistical Analysis

To be able to decide whether or not one or more species are present in the *imperialis* and *africana* groups, statistical argumentation can be helpful. Especially when dealing with this kind of complicated groups. Therefore, after all the measurements were finished I performed a Principal Component Analysis (PCA) using R (Team, 2005) (Appendix 2 for exemplary script).
PCA is regularly used in morphometric research since it finds which characteristics in your dataset are of significance. These characteristics are found by looking at the amount of variance of the data they explain. The more important the character (or variable), the more variance is explained by it.
In PCA, a 3D scatterplot of all data points is formed by an algorithm. Within this cloud of points, the algorithm searches for the longest axis. This first axis explains the most variance. The second axis is placed perpendicular on the first axis and so on. Every variable has a weighting, and this weighting indicates how much this variable contributes to the axis (Dytham, 2011). The results are then visualised in a biplot.

When performing a PCA in R, you first make a CSV file of your data. This file is loaded in R, and the specimens and characteristics are specified. Then, you perform the analysis by giving the commands.
Since the dataset contains data with a number of different units, I specified that the scale is TRUE. The data is now detrended. The command 'summary()' then gives the final overview of results. Here you can see how many variance is explained by the characteristics. Last, the command for a biplot is used to visualise the results. In the biplot, the characteristics are represented by arrows and the samples (specimens in this case) are dots.

To optimally perform the statistical analysis, I divided the data that I collected in the Excel file over two separate databases (one for the *imperialis* group and one for the *africana* group). This is because of the way R (the analysing program I used) deals with missing values in the test I performed. R namely either deletes the entire sample row with the missing value, or the missing measurement is replaced for a substitute value. Therefore, it was unfortunately not possible to analyse these two groups together.
In addition, it turned out that all measurements of one of the groups I formed using morphological methods (see previous paragraph) were excluded from the analysis. A discussion on this subject can be found in the Discussion and Conclusion of this chapter.

4.3 RESULTS

4.3.1 MORPHOLOGY

4.3.1.1 IMPERIALIS GROUP

To answer the first research question I mainly performed a literature study. In addition, I looked into a number of characteristics to see whether there is a difference between what now is called *V. imperialis, ochyrea* and *lujae* or not.

There are four publications that describe *V. imperialis, ochyrea* and *lujae*. The first is the typefication of *V. imperialis* by Kraenzlin in 1896 (Kraenzlin, 1896). In the drawings accompanying the text describing the morphology (Figure 8, top picture), it can clearly be seen that the plate of the lip is trilobal and quadrangular when loosened from the column (red arrow in the picture). In natural position, the plate of the lip is not folded and forms a quadrangular plate (green arrow in the picture). Also, the bracts are positioned rather densely on the inflorescence and multiple flowers are opened at the same time.
In the publication of Portères (1954), the species *imperialis* and *lujae* are still described as separate species based on differences in the shape of the lip. In the drawing that visualises these species (Figure 8, lower left picture), the two of them are pictured together. The top flower (indicated as *V. lujae*, blue arrow) has a pointed lip and the other flower (indicated as *V. imperialis*, purple arrow) does not.
In the Flore du Cameroun by Szlachetko & Olszewski (1998), *V. imperialis* is depicted by the drawing as shown in the lower middle picture of Figure 8. In this picture, it can be seen that they interpret *imperialis* to have a pointed lip.
The lower right picture of Figure 8 shows a drawing of the new species *V. ochyrae.* It is described in this publication as being distinctly trilobal.
Last there is the original publication of *V. lujae* by De Wildeman in 1904, published in the 10th edition of the Bulletin de la Société Belge d'études coloniales, of which Marc Sosef was able to provide me with a copy. Unfortunately, no (clear) picture is available in this article, but a number of characters are listed that separate *V. lujae* from both *V. planifolia* and *V. imperialis.*

In the morphological part of the research I looked at a number of characteristics as described in the Materials and Methods of this chapter. A number of these characteristics is solely used in the statistical analysis which will be discussed later in this paragraph. These are characters as length, width and angle of the lip.
The other characteristics I only looked at to detect whether they are suitable to divide the available flowers over groups 'by eye'. One of these is the attachment of the flower to the column. In some flowers, it seemed that the side flaps of the lip were attached to a large part of the column. Only the tip of the column is still shown (Figure 9, left picture). In other flowers, this was not the case and a much larger portion of the column was free.
I also looked at the shape and positioning of the bracts on the inflorescence of *V. imperialis, V lujae* and *V. ochyrae* because they are described to be different (middle and left picture of Figure 9). Indeed differences can be found and the specimens were divided over two piles by eye. In the one pile, the bracts are positioned really quite dense on the inflorescence. In the other specimens, the positioning of bracts is less dense. Also, in the latter the bract appear to be smaller.

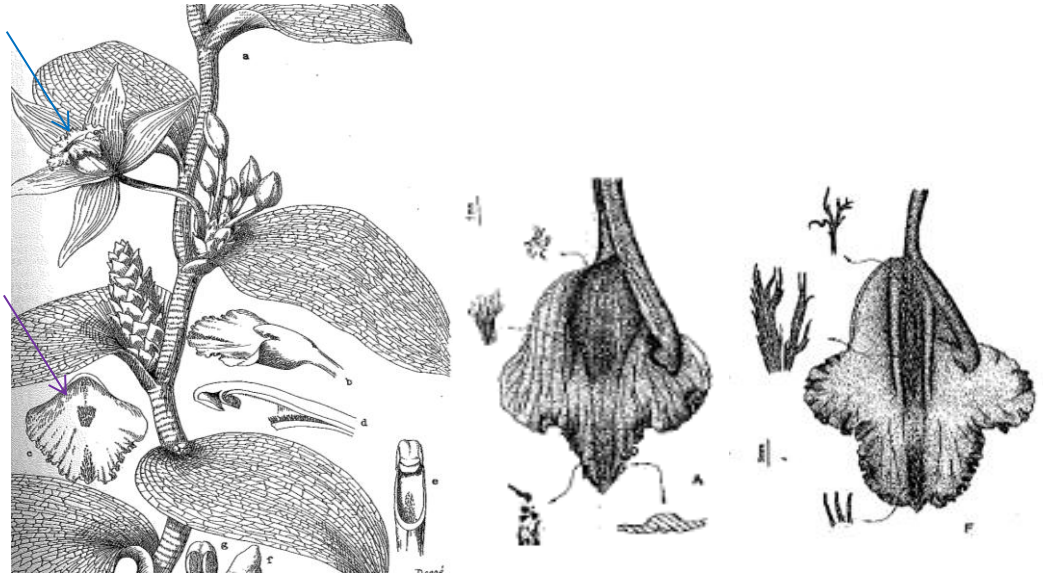

FIGURE 8. DRAWINGS ATTACHED TO THE PUBLICATIONS OF KRAENZLIN (TOP, *V. IMPERIALIS,* 1896), PORTÈRES (LOWER LEFT, *V. IMPERIALIS* AND *V. LUJAE*, 1954) AND SZLACHETKO & OLSZEWSKI (LOWER MIDDLE AND RIGHT, *V. IMPERIALIS* (MIDDLE) AND *V. OCHYRAE* (RIGHT), 1998)

FIGURE 9. LEFT: EXAMPLE ATTACHMENT OF THE LIP TO THE COLUMN (...); MIDDLE: EXAMPLE OF POSITIONING OF BRACTS IN *V. IMPERIALIS* (PERMOETEN 391); RIGHT: EXAMPLE OF POSITIONING OF BRACTS IN *V. LUJAE* (GERARD 5078).

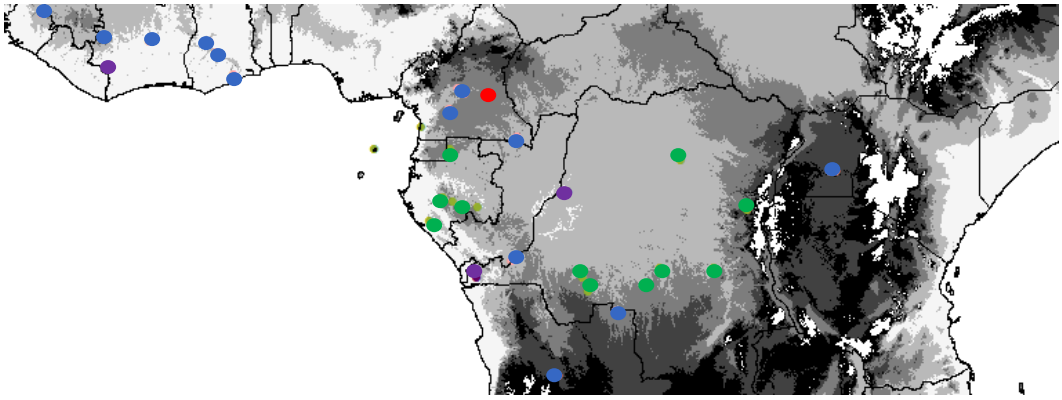

FIGURE 10. DISTRIBUTION MAP OF THE IMPERIALIS GROUP. COLOUR INDICATES SPECIES: *V. GRANDIFOLIA* (GREEN), *V. IMPERIALIS BY SZLACHETKO & OLSZEWSKI* (BLUE), *V. OCHYRAE BY SZLACHETKO & OLSZEWSKI* (RED) AND *V. POLYLEPIS* (PURPLE).

Theo Damen entered all data from the labels of all available specimens in the Brahms database and found coordinates for them.
As can be seen in Figure 10, *V. imperialis* sensu *Szlachetko & Olszewski* has quite a wide distribution ranging from Sierra Leone all the way south to Angola and as far to the east as Uganda. *V. grandifolia* is distributed from Sao Tomé to the Democratic Republic of the Congo. *V. ochyrae by Szlachetko & Olszewski* is here found in only one location in the middle of Cameroon. Also *V. polylepis* can be found on the African continent in Democratic Republic of the Congo and Ivory Coast.

4.3.1.2 AFRICANA GROUP

The first characteristic that I found was whether or not a callus is present at the rear of the lip (left picture of Figure 11). This callus is an augmentation on the back of the lip behind the fans and appears to differ in size. The presence of the callus seems to be arbitrary since of two flowers from the same collection, on only one a callus was present (collection *Lebrun 3241*).

After looking at many flowers, I was able to form five morphological groups by eye from the flowers I kept on alcohol (Figure 12, Appendix 3). What distinguishes the first group from the others (top left and top right picture in the figure), is the strong broadening of the lip positioned closely towards the apex. A broadening is present in some of the other groups, in which case it is positioned more to the middle of the lip. In addition, the broadening present in this first group appears to be more of a continuation of the width of the lip towards the apex, resulting in a quite rectangular lip form.
Comparatively, the second morphological group has a more ovate lip shape as can be seen from above in the middle left picture.

The third group has a lip that is similar to that of the second group, but the side of the flower is quite different. The tissue at the side of the lip that connects the lip with the column is quite straight in the second group. It starts quite close toward the end of the column and goes down ending just before the fan like callus in the middle of the lip (red line in picture). In the third group however, this slip is much more withdrawn towards the base of the flower at the lip (blue line of middle right picture in the figure).

The fourth group is also quite similar to the second group, but a difference can be found in the shape of the lip. In these flowers, the lip is much straighter and not as ovate as that of the second group (lower left picture). For the rest, the flowers in this group are rather similar.

In the fifth group the lip is also quite narrow and the lateral lobes are also withdrawn just like in the third group. In addition, the angle between the column and the lip is much smaller than in the other cases (lower right picture).

FIGURE 11. LEFT: EXAMPLE OF QUITE LARGE CALLUS (LEBRUN 1948); MIDDLE: CALLUS IS PRESENT (LEBRUN 3241); RIGHT: CALLUS IS ABSENT (LEBRUN 3241).

FIGURE 12. OVERVIEW OF THE FIVE GROUPS FORMED 'BY EYE'. TOP LEFT: ; TOP RIGHT: ; MIDDLE LEFT: ; MIDDLE RIGHT: ; BOTTOM LEFT: ; BOTTOM RIGHT.

I also looked into the morphology of the leaves of both the *imperialis* and the *africana* group. As described, I divided all the herbarium specimens present by eye over different piles. I ended up with 23 different piles. In Figure 3 I present a selection of these groups. As can be seen in the figure, there are quite a lot of different leaf shapes. Some are almost round, others more oval and some are lanceolate. Within these shape outlines, there is also variation in size. For example, the top left an middle left picture are both lanceolate, but the leaves in the top left picture are twice as large as the leaves in the middle left picture.

Also, there is quite some variation in leaf shape in what previous researchers determined to be of the same species. For example, the middle right and lower left picture (*J.J. Bos 5992 and Barker 1265*) are both determined to be *V. crenulata*.

FIGURE 13. OVERVIEW OF THE DIFFERENCES IS LEAF SHAPE. TOP LEFT: LEEUWENBERG 5465; TOP RIGHT: LOUIS 3599: MIDDLE LEFT: VERMOESEN 1869; MIDDLE RIGHT: J.J. BOS 5992; BOTTOM LEFT: BARKER 1265; BOTTOM RIGHT: GERARD 5078.

As described earlier, I searched for some additional characteristics within the specimens of the relevant morphological groups by eye.
While looking at the peduncle, I made only one pile. There is thus no variation within this character.
I also looked at the inflorescences. Figure 4 shows a number of the inflorescences I looked at. I divided the set of specimens over three piles: one pile with branched bracts (lower left), one with bracts that were not branched at all (top left) and one with both branched and unbranched on the same individual (top right).
There can also variation be found within the piles. The lower right picture shows a second inflorescence from the unbranched pile. This inflorescence is quite long and narrow, while the inflorescence as shown in the top left picture (from the same pile), is much shorter and thicker.

FIGURE 14. VARIATION IN INFLORESCENCES.

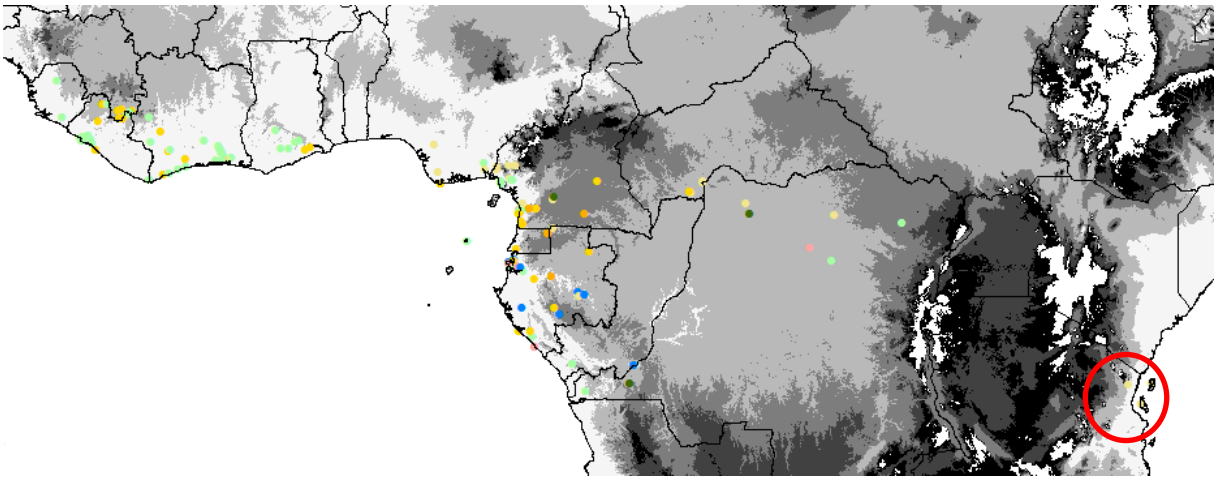

FIGURE 15. DISTRIBUTION MAP OF THE IMPERIALIS GROUP. COLOUR INDICATES SPECIES: *V. ACUMINATA* (PINK), *V. AFRICANA* (BRIGHT YELLOW), *V. CRENULATA* (LIGHT GREEN), *V. CUCULLATA* (ORANGE), *V. HETEROLOPHA* (BLUE), *V. RAMOSA* (DARK GREEN AND LIGHT YELLOW).

Figure 15 shows the distribution of the species present in the *africana* group. As can be seen in the figure, all species quite cluster together around the same region. Their distributions overlap with a few outlyers, ranging from Liberia east and into the Democratic Republic of the Congo. *V. crenulata* and *africana* have quite a wide distribution, spanning the entire area. Quite a large gap seems to appear in the distribution of *V. ramosa*: it occurs inland on the west side of Cameroon, but is also found along the coast of Tanzania. *V. heterolopha* is mainly clustered around Gabon and Congo.

4.3.2 Statistical Analysis

Here, I will present the results of the statistical analysis. First those for the *imperialis* group, then for the *africana* group.

Appendix 4 shows the results of the PCA done on the *imperialis* dataset. As can be seen in the table, 75.91% of the total variance is explained by PC1 and PC2. The first axis is represented by the characteristic 'angle_left' (angle of the left side of the lip with midline) with a value of -0.4382548. However, the weightings of the characteristics 'total_length' (length of the entire flower) (-0.4329647) and 'column_length' (length of only the column) (-0.4362589) are quite similar. So a combination of these three works well to explain the first axis. The second axis is most explained by the character 'column_width' (width of the column) which has a weight of -0.5652310. These results are also shown in the top left biplot of Figure 16.
As can be seen from the figure, the characteristics 'angle_left' and 'angle_right' (angle of the right side of the lip with midline) seem to cluster together with m_length. Also lip_lenght (length of the lip, from apex until end of the column) and total_lenght cluster together.

For the *africana* group, I performed a number of PCAs that differ in the composition of characteristics and how the dataset was assembled. Biplots of these three PCAs are also shown in Figure 16 (top right, bottom left and bottom right).

The first PCA comprises a selection of characteristics that combined covered the data in the best way and also includes a number important characteristics that were indicated as important beforehand (for explanation and discussion of this problem, see 'Discussion and Conclusion' section section of this chapter). These characteristics and the results of the PCA are listed in Appendix 4. As can be seen in the table, 57.01% of the variance is explained by the first two axes. Adding the third axis increases the amount of explained data to 70.99%. The first axis is represented by the character LCT (total length of the column) with a weight of 0.4808846. The character A2 (second angle within the column) with the weight 0.55104330 seems to represent the second axis. Last, the third axis is represented by the character A3 (angle between the lip and column) with a weight of -0.59612249.

The second PCA comprises almost the same combination of characteristics, but I left out the obscure character 'fans'. This resulted in the biplot shown at the bottom left in Figure 16 and the results are shown in Appendix 9.10.3. With the first two axes, 62.72% of the data is explained. The first axis is again represented by the character LCT with a weight of 0.4780531. The character A1 (first angle within the column) here explains the second axis with a weight of 0.56216422.
Adding the third axis increases the percent of explained data to 74.60% and the character that explains it is again A3 with a weight of 0.68577778.
I performed the third PCA on a dataset with the same characteristics as the second PCA, but here the number and composition of specimens differed from the second PCA (see 'Discussion and Conclusion section of this chapter for explanation). Of this PCA, the results are shown in Appendix 9.10.4 and the lower right biplot of Figure 15. In this PCA, the first two axis explain 63.84% of the data. Again the first axis is explained by the character LCT with the weight of 0.4769624. The character A3 explains the second axis with a weight of -0.54151089.

With the addition of the third axis, the amount of explained data increases to 74.53%. The character WLT (total width of the lip) explains this axis with a weight of -0.64984080. The character A3 also has an influence here, since its weight is 0.63869005.

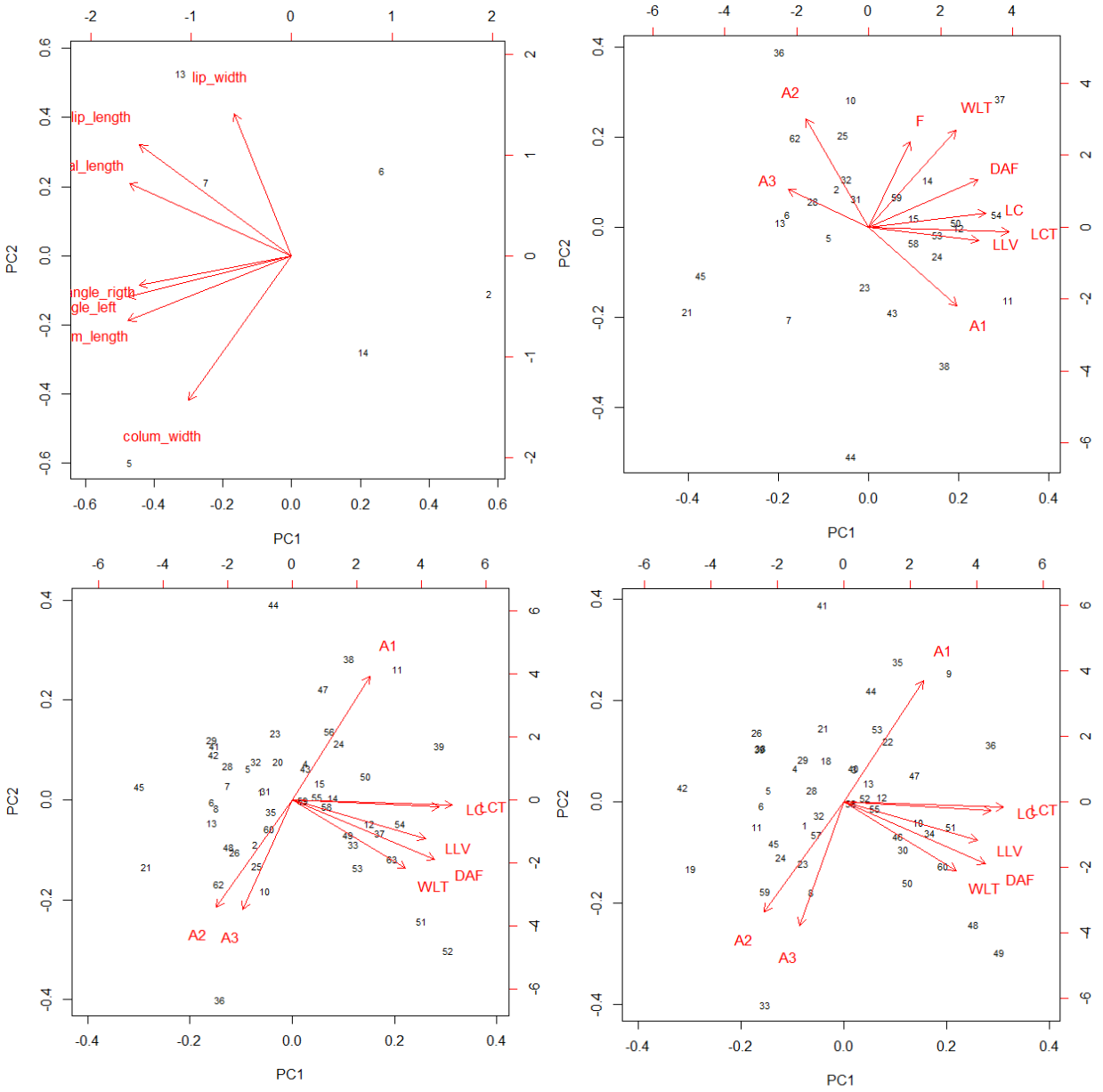

FIGURE 16. PCA BIPLOT OF IMPERIALIS (TOP LEFT), AFRICANA WITH FANS (TOP RIGHT), AFRICANA WITHOUT FANS (BOTTOM LEFT) AND AFRICANA WITHOUT FANS AVERAGE (BOTTOM RIGHT).

4.4 Discussion and Conclusion

Here, I will discuss the results as presented in the previous paragraph per research question. At the end, a number of additional matters will be discussed.

4.4.1 Imperialis group

The first research question addresses the issue with the confusion between species in the *imperialis* group. The first hypothesis is that Szlachetko and Olszewski wrongly described the new species *ochyrae* while this species was already described as *imperialis* by Kraenzlin in 1896 (Kraenzlin, 1896). Based on the literature research I performed and the drawings available of the interpretation of the species by the different researchers, I can say this indeed is the case. The description of what Szlachetko and Olszewski call *V. ochyrae* and what is described earlier by Kraenzlin and Portères as *V. imperialis* is so similar, I state they are one and the same species and the first hypothesis of this question is thus accepted.

Then there is the question which species is then mixed up with what Szlachetko and Olszewski call *V. imperialis*? Because there has to be a species they exchanged for their version of *imperialis*. This is sought-after in the second hypothesis where I hypothesised that the species described by Szlachetko and Olszewski as *imperialis* is in fact a previously described species called *V. lujae*.
Evidence for this can be found in the publication of Portères of 1954 and the description and typefication of *V. imperialis* by Kraenzlin (1896). Portères describes both *imperialis* and *lujae* and states that the difference between them is whether the lip is tri-lobed or not. According to Portères, being tri-lobed results in a lip plate that is quadrangular (as in *imperialis*) while not having a tri-lobular lip results in a pointy lip (like *lujae*). The drawings that accompany his publication can be found in Figure 8.
Striking about this drawing is that it is so similar to the one of the original *imperialis* publication by Kraenzlin. Compare the flower pointed out by the purple arrow in the publication of Portères with the red one in the Kraenzlin publication and spot the differences.
In contrast to the species addressed by Kraenzlin and Portères, Szlachetko and Olszewski describe *imperialis* as having a pointed lip and being not tri-lobed. Thus based on this literature, I conclude that indeed the species that is called *V. imperialis* by Szlachetko and Olszewski (1998) is in fact *V. lujae* as described by Portères (1954).
In this confusion, there are thus only two species at play. One called *V. lujae* that was made to disappear into synonymy and confused with *V. imperialis* by Szlachetko and Olszewski. And the real *V. imperialis* that was thought to be a new species called *V. ochyrae* because its name had wrongly been labelled at the description of another species.

In addition to this literature study, I performed morphological research to form my own opinion about these species as described above. In the end, this thus resulted in a not intended research whether the real *V. imperialis* is actually the same as the real *V. lujae*. Unfortunately in this group, only a small amount of specimens made it to the statistical analysis (top left biplot of Figure 16 in this chapter). How this could be possible is discussed below in more detail under 'General comments', but the same principles apply here. Briefly: it was not possible to measure all characteristics for all specimens and as a result a large number of accessions is excluded from analysis.
As can be seen in the biplot, this PCA does not give any information since there are only six specimens left in the analysis. In total, I studied 28 flowers of almost 70 specimens. It is thus quite disappointing that only these six have made it to the final analysis. No conclusions can be based on an analysis of so few measurements.
However, while studying these flowers I did get the chance to investigate the most important difference between these species: whether the lip is quadrangular or pointy. I indeed can make a clear separation between individuals with flowers that are pointy and those that have a quadrangular lip shape. And there is no overlap between these groups. Sometimes this is quite hard to see because the sides of the lip are rolled-up or torn. But with some patience in the preparation of the flowers you can indeed determine whether the lip is actually quadrangular.

I also looked at a number of vegetative characteristics as explained earlier in this chapter. There turned out to be a difference between the bracts of *imperialis* and *lujae*. Those of *imperialis* are rather tightly packed together on the inflorescence (Figure 9). The bracts of *lujae* are not that close together. In both cases, the bracts are quite large compared to those of the *africana* group.
With these differences, I find no reason to go against the findings of previous researchers and conclude *imperialis* and *lujae* indeed are different species.

4.4.2 AFRICANA GROUP

The second research question asks 'How many (sub)species are present in the *V. africana* group' as described by Soto Arenas and Cribb (2010). The hypothesis states that all the eight species that have been described indeed are species.

In the morphological part of the Results, I explained that I found five distinct groups of flowers on alcohol. These groups are quite easy to separate from each other based on a few, always present, characteristics. Appendix 3 shows an overview of the flowers (numbered with collector name and number and group number) for each of these five groups. As can be seen from the table, flowers that I placed in different groups based on their appearance have the same collector-number combination. For example, I placed flowers belonging to the collection *Lebrun 2493* in groups 2 and 3, flowers belonging to the collection *Bos 4195* ended up in groups 2 and 4 and those of the collection *Leeuwenberg 3054* in groups 3 and 5. So multiple flowers from one individual plant can be placed in different groups based on their morphology. The only exception is morphological group number 1, which is the only group that has no overlap with the others. Based on this result, I can say there are only two entities in the *africana* complex. One entity that comprises morphological group number 1 (*V. heterolopha*), and the other entity comprises morphological group number 2,3,4 and 5.

This conclusion can unfortunately not be directly backed up by the results of the statistical analysis. The PCA of Figure 15 shows a nice scattered cloud of flowers, indicating the flowers cannot be separated based on the measured characteristics.
Not all flowers that are placed in a morphological group are taken into the analysis because some of them were damaged. When a flower is damaged, some characteristics might not be measured. This results in a gap in the database: a missing value. R performs PCA in such a way that it does not allow for empty fields in the datasheet (missing values). Therefore, all rows in the database that contain missing values are omitted from the analysis. As a result, the data file that comprised measurements for 130 different flowers ranging all five morphological groups, was reduced to a file with zero flowers because for each of the flowers a value is missing over the whole range of characteristics. In order to keep as many flowers in the analysis, I first removed a number of characteristics which contained too much missing values. Of the original nineteen characteristics, in the end I only used nine.

The result that there are no separate groups found in the PCA does not necessarily mean there are no such groups. I started without any knowledge of the morphology of the flowers I was about to research. This is a good way to remain objective and to prevent a possible bias towards certain characteristics you try to find because they are described in the literature. On the other hand, you end up with characteristics that may seem of some importance, but turn out to be not that interesting. In my case, I ended up with nineteen characteristics I thought could be of interest (including the usual length and width measurements). I used none of these to form the five morphological groups. The one characteristic I used to separate morphological group 1 from morphological groups 2, 3, 4 and 5 by eye I did not think of to measure at the time I started. There are thus no measurements of this particular character and it is also not included in the PCA. This is quite a pity. In the future, I will adopt the method that is more commonly used in taxonomy: start by reading the existing literature, looking at the plants and research their described and newly found differences 'by eye'. If there are characteristics that divide the plants over different piles, measure those characteristics. In this way, you

actually measure characteristics that are important in the description of the entities. Of course, you could perform additional measurements on the more general characteristics as certain length and width measurements. But these should be taken alongside a set of forethought characteristics.

For quite a number of specimens, I was able to measure more than one flower from the same plant. This could give a possible bias in the statistical analysis, since flowers growing on the same plant have the same influence from environmental conditions (as the weather and soil composition) and genetics. The easiest solution to prevent any possible bias is to take average values for those specimens of which multiple flowers were measured. But then, a lot of variation could possibly be removed. The second possibility is not to perform PCA, but use an alternative correlation test called Canonical Variance Analysis or Canonical Component Analysis (Dytham, 2011). This test works in much the same way as regular PCA, but instead of taking all the accessions (or rows) separately, you can assign them to groups. These groups are then tested in the normal PCA way, This is a good way to for example compare between populations while keeping the variation within every population.
To decide which of the above named alternatives would be would be best for my data, I performed a preliminary PCA on my already dressed-down dataset. I then checked which of the specimens were not taken in the analysis and removed them from the data file. For the remaining specimens I checked whether they possibly belonged to the same collection. This turned out to be the case in only four out of 60 accessions. So of two specimens, two flowers each were measured. When I checked the measurements, I did not find a large variation between the flowers for the specimens. Therefore, I took their averages and repeated the PCA. As can be seen in Appendix 4., the percentage of variation explained increases with just over 1%. There is thus not a lot of difference when you take the average values in the few cases there is overlap in specimens. This in combination with the fact that CCA is normally used to compare not such a large amount of groups with so little measurements, led me to the conclusion that averaging the values can be allowed in this case.

In addition, neither in the morphological groups or the statistical analysis all entities named in the hypothesis are taken into account. In the morphological groups, only specimens identified as *V. crenulata, ramosa, cucullata, acuminata, heterolopha* and *africana* are included. Since there are only one or two specimens of the other entities (*V. hallei* and *zanzibarica*) available, I found it not possible to properly incorporate them.
Why not all species are included in the statistical research is, as in the *imperialis* group, a result of the analytical program R and will be discussed below.

All in all, I find I cannot form any solid conclusion based on the present information and thus neither accept nor reject the hypothesis.
However, from the 'gut feeling' I got from looking at these flowers for four months, I can say that the only entity that is easily recognised in this group is *V. heterolopha*. The flowers of this species are clearly distinguishable from the others, because of the broad shape of the lip.
There indeed are differences to be found between individual flowers of the other entities, but as described above, in the statistical analysis I did not find any characteristics separating the specimens in clear groups.

4.4.3 General comments

As briefly stated above, the large amount of missing values is not a result of absence of the character that was to be measured. It is a direct result of the bad shape of many of the flowers I examined. This is true for flowers of both research groups.

If the characteristics were absent, I would have entered the value '0', but quite often the flowers were torn or damaged in such a way that I found it impossible to accurately take measurements. In some flowers, for example, the sides of the flower tube was cut open to examine the inside of the tube. However, in cutting open the side of the tube, the whole structure of the flower is lost. It is therefore no longer possible to measure the length of the lip that is visible from the side of the tube until the acumen, since the side of the tube is missing. Also, since there is no longer structure in the flower, the angle between column and lip cannot be measured. The final dataset is thus a trade-off between the number of characteristics and amount of missing values.

This issue with missing values is quite a problem since a lot of potential valuable data is excluded from the analysis. The easiest solution would be to make sure you measure every character for every accession. But that is thus more easily said than done.

Now, I excluded the characteristics with a large amount of missing values from the analysis to keep as many flowers in the dataset as possible.

As described in the Material and Methods of this chapter, I wanted to use the program tpsDig 2 for measuring specimens. tpsDig 2 can be quite useful when performing an extensive morphological research. Especially when measuring skulls or the length of a stem with a clear beginning and end. However, as can be seen in the screenshot of the program in Figure 17, the flowers I worked with have a fleshy texture. This makes it difficult to accurately define the transition of one structure into the other in a photograph. For example, where to place the mark defining the fusion of the lip and column in this photograph?

Also, it turned out to be difficult to take pictures of the flowers in a way there would be usable for the program. I took the pictures through the binoculars, but it was not possible to fasten the camera to these binoculars. So I took the pictures with one hand, while keeping the flower in place with the other to make sure the picture could be taken from the right angle.

In addition, for the program to function optimally, the object has to be in the same position at every picture. This is not possible with these flowers, since not all the landmarks can be set in one picture. Multiple pictures are needed to cover all the landmarks and as a result you have to position the landmarks in every picture instead of the program automatically setting them for you. Then, since some flowers are larger than others, no standard enlargement could be set. This means that for every picture you in addition have to set a scale.

This combined made that the program tpsDig2 is not convenient to work with when using this kind of material. Therefore, all measurements were taken by hand using a normal ruler.

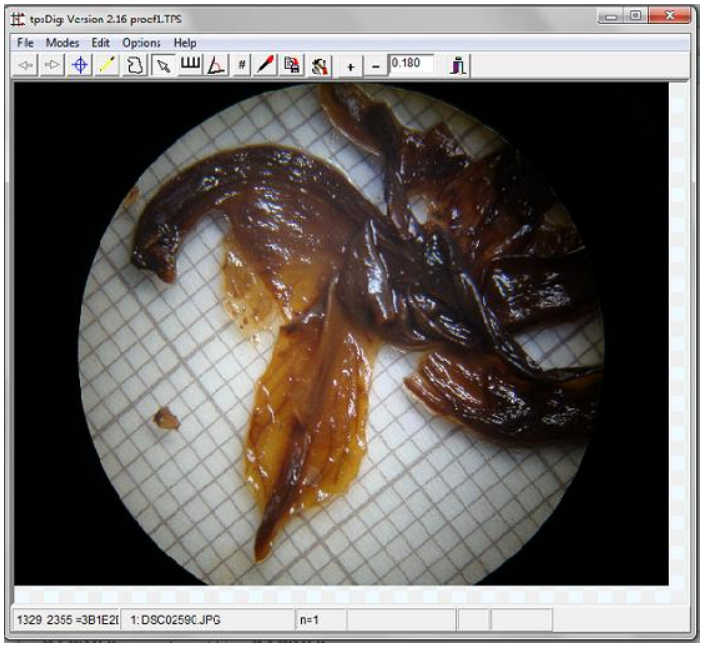

FIGURE 17. SCREENSHOT OF TPSDIG 2.

As described in the Materials and Methods of this chapter, it could be quite handy to be able to distinguish between species based on vegetative characteristics. In addition to the method I have now used (dividing the specimens by eye), I had planned to also form a statistical basis for this. To this end, I have scanned all specimens using the scanner from the digitalisation project of Naturalis Biodiversity Center. Using these scans in the Herbarium Leaf Finder program (Corney, Clark, Tang, & Wilkin, 2012) for Matlab, the length, width and other characteristics of every leaf can be measured more easily than when done by hand.

Due to licensing issues I was not able to perform these analyses myself. Fortunately, Kris van 't Klooster of the Cell Biology department of Wageningen UR was willing to assist. After some start-up problems, the program did work and a number of scans were successfully analysed. However, because the scans are of such high quality, the analysis takes quite a lot of time. Therefore, we now only performed a preliminary study on three scans to explore the program. With the limited amount of results we now generated, the HLF program indeed seems quite promising for future research. A large amount of data can be generated quite easily when using this program. You only need to make sure there is a picture of a good exemplary leaf available since the program uses it to find the leaves on the herbarium sheets by matching the general outline of the example to structures on the sheet. When the sheets are analysed and candidate leafs are selected from them, the program presents them and all you have to do is select the ones that you are interested in. Then, Matlab automatically measures the characteristics and you can export the results to an Excel file.

With this program, not only leaf characteristics as length and width can more easily be measured, but there are also options to explore for example the length of the acumen and the shape of the leaf can be described by algorithms. Also, the program can use algorithms to describe whether a leaf is crenature or not and how much so.

5. DNA-BASED PHYLOGENETIC INFERENCE

In this chapter, I address the third research question:

3. Can (part of) the phylogenetic hypothesis of Bouetard *et al.* (2010) be complemented and optimised with additional *Vanilla* species, especially those from the *V. imperialis* & *africana* groups as described by Soto Arenas & Cribb (2010)?

5.1 INTRODUCTION

Since the coming of molecular systematics, much research has been done into the genetics and evolution of the higher taxonomic levels of the Orchidaceae. Examples of this are the identification of the sister clade of *Vanilla*, *Pseudovanilla*, and its position within the Vanilloideae with the use of plastid genes *psbB* and *psbC* by Cameron & Molina (Cameron & Molina, 2006). Cameron has also used the gene *psaB* to investigate and identify the intergeneric relationships of the different genera found in the Orchidaceae (Cameron, 2004, 2009). Also *matK* and *rbcL* data have been used (Freudenstein et al., 2004).
More recently, research has been done into the (phylo)genetics of *Vanilla.* The origin of *Vanilla tahitensis*, for example, has long been a puzzle. There are no wild *Vanilla* species known from the Australia/Pacific Island region, but *V. tahitensis* is grown in cultivation there. Analysis of the nuclear ribosomal ITS and plastid *trnH-psbA* regions has revealed that the species is a hybrid of *V. planifolia* and *V. odorata* (Lubinsky, 2008b). In this study, it was determined that *V. planifolia* is the maternal and *V. odorata* is the paternal line that combined form this commercially used hybrid grown on the Tahitian islands (Bory et al., 2007; Cameron, 2011b; Ecott, 2004; Lepers-Andrzejewski et al., 2011; Lubinsky et al., 2008). Also, the genetic background of *V. tahitensis* is found to be reflected in its morphology since the plant forms a nice bridge between the parent species integrating key characteristics of both of them (Cameron, 2011a).

Also, microsatellite markers have been developed for *V. planifolia* that were transferable to other species. More recently microsatellites were used in a study of *V. humblotii*. These markers can help in further research into the hybridization of *Vanilla* and in research on population genetics (Bory et al., 2008; Gigant et al., 2011). Bory *et al* (Bory et al., 2008) also discovered that *Vanilla planifolia* forms naturally occurring triploid and autotetraploid types in Reunion Island. These types of polyploidy can be linked to phenotype variation.

In 2003, Soto Arenas published a phylogenetic tree of *Vanilla* based on *rbcL* sequences separating the genus in three clades: 'Membranaceous', 'Old World & Caribbean' and the 'America fragrant' clade. Here, it became clear that the species found in the Caribbean and Asia have their origin in African clade. Bouetard *et al.* published a phylogenetic tree of *Vanilla* species in 2010. They analysed the *psbB*, *psbC*, *psaB* and *rbcL* genes of chloroplast DNA of 47 *Vanilla* accessions from all around the world and confirmed that *Vanilla* originates in South-America, radiated to Africa and from there dispersed further to Asia and back to the Caribbean. Just a few months later, Soto Arenas and Dressler published a revision of the Mexican and Central American *Vanilla* species (Soto Arenas & Dressler, 2010). They used the nuclear ribosomal ITS region to determine the relationship between the *Vanilla* species growing in these areas.
There are a few weaknesses in these publications. The first one of Soto Arenas only includes the *rbcL* gene, which is a bit weak to base a species level phylogenetic tree on especially since it is a chloroplast gene which is only maternally transferred. This phylogenetic tree was further developed by Bouetard *et al.* who added additional species and used more genes, albeit chloroplast genes which can result in a different phylogenetic tree compared to nuclear DNA which is both maternally and parentally transferred. The last phylogenetic tree of Soto Arenas & Dressler was based on nuclear ITS rDNA, which is a huge improvement, but only includes American and Mexican species. Therefore, a complete (combined nuclear and plastid DNA based) phylogenetic hypothesis still needs to be produced (Álvarez & Wendel, 2003).

Of course, that is quite an undertaking for a MSc thesis. Therefore, we set the more modest goal to try to optimise the phylogenetic tree of Bouetard *et al* (2010) (being the third research question of this project). I attempted to do this in two ways. Since a number of important species is missing from the phylogenetic tree, I tried to implement these specific species in the phylogenetic analysis using historical DNA obtained from herbarium samples.

While working on this project, we decided to start a second project alongside it. Initially, we wanted to use the material of Bouetard *et al* (2010) to extract and sequence the 5.8S rDNA gene on the nuclear ribosomal ITS region to link the phylogenetic tree of Soto Arenas and Dressler (2010) with the one of Bouetard *et al* (2010). Unfortunately, I could not acquire the necessary material. Therefore, we decided to gather as much silica dried material as possible and use the same primers as Bouetard *et al* (2010) used for extraction and DNA sequencing. In this way I can still expand the Bouetard *et al* phylogenetic tree and also explore the possibilities of a standard barcode for certain species.

This genetic part of the research is thus again divided in two sets: one historic DNA and one 'regular' DNA research line. But they are still interwoven and complement each other. Therefore, I will discuss them in the same chapter alongside one another.

5.2 Material and Methods

As can be seen in Figure 18, only a small number of Continental African species is included in the phylogenetic tree of Bouetard *et al.* (2010) and quite a number of species is thus missing. In addition, many vouchers have not been identified and two major morphology based groups (Soto Arenas & Cribb, 2010) have not been sampled at all. Therefore, quite a lot of work can still be done on this phylogenetic tree.

To start, the vouchers of the sequences used in the phylogenetic tree have been asked on loan. With these vouchers, a possible definitive identification can be made for all the yet unidentified sequences in the phylogenetic tree. This would already take away some of the ambiguities in the phylogenetic tree.

Second, the species that are still missing from the phylogenetic tree had to be analysed in as much the same manner performed by Bouetard *et al*. in their experiment as possible. This ideally would have concerned the species *V. chalotii*, *nigerica* and *seretii* from the *V. chalotii* clade, *V. acuminata*, *cucullata*, *hallei*, *heterolopha*, *ramosa* and *zanzibarica* from the *V. africana* clade and *V. grandifolia* and *ochyrae* from the *V. imperialis* group. In this way, all the continental African clades are fully added to the phylogenetic tree.

Since different researchers have negative experiences with the extraction of orchid DNA from herbarium specimens (Gravendeel, personal communication; pilot study, Biosystematics group DNA lab), this method was not further investigated in this project. However, an Ancient DNA lab is available at Leiden University. In this lab, special protocols have been developed for the extraction of ancient and historic DNA residues and here the extraction and PCR amplification of orchid DNA from herbarium specimens has been done before (Gravendeel, 2010).

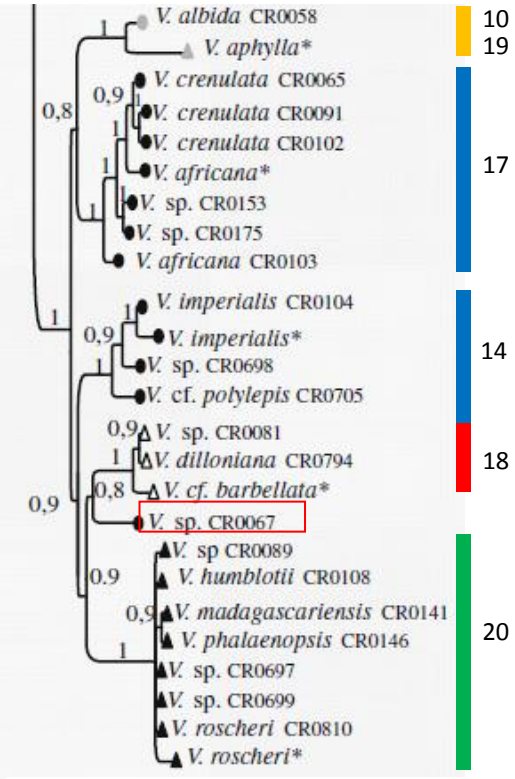

FIGURE 18.5 PHYLOGENETIC TREE OF BOUETARD ET AL. (2010) WITH THE INFORMAL GROUP NUMBERS OF SOTO ARENAS & CRIBB (2010)(10: V. ALBIDA GROUP, 14: V. IMPERIALIS GROUP, 17: V. AFRICANA GROUP, 18: V. BARBELLATA GROUP, 19: V. APHYLLA GROUP, 20: V. PHALEANOPSIS GROUP). COLOUR INDICATES GEOGRAPHICAL REGION: ASIA (YELLOW), CARIBBEAN (RED), CONTINENTAL AFRICA (BLUE) AND MADAGASCAR (GREEN) (AFTER BOUETARD *ET AL.* (2010).

Below, first the methods for the historic DNA and regular DNA extraction, amplification and sequencing will be explained. Thereafter, the methods I used in the phylogenetic analysis of the sequences will be explained.

5.2.1 Historic DNA methods

Recently, Gravendeel *et al*. were able to sequence the entire chloroplast genomes of twelve orchid species and a complete nuclear genome of an *Arabidopsis thaliana* herbarium specimen using the protocols mentioned above. They could identify fast evolving regions in the chloroplast *rbcL* gene and developed new primers corresponding to various regions. Since these primers prime on short (ca. 100 bp long) regions and only need a small amount of DNA template to work, they are ideal for the type of DNA obtained from herbarium specimens. This is because the samples, even when obtained in the Ancient DNA lab, are expected to contain only highly degraded DNA with fragmented pieces shorter than 400 bp.

Together with Barbara Gravendeel I collected suitable samples of the relevant species (Table 1). For each species we selected one herbarium specimen and hereof took a square centimetre of leaf tissue for analysis after obtaining permission of the administering herbarium.

In the Ancient DNA lab in Leiden and under supervision of Aline Nieman, these samples were ground in a sterilised mortar after being frozen in liquid nitrogen. Then, we extracted the DNA in two ways. One is based on the original CTAB procedure of Doyle (Doyle, 1991) and the other is based on a silica method developed by Hofreiter (Hofreiter et al., 2000; Hofreiter, 2001) (Appendix 5 and 6). Most important to note is that the main difference between these extraction protocols and the use of 'normal' commercial kits is that soap and silica were used to precipitate the DNA fragments rather than membranes in plastic columns (van Geel, et al., 2011; van Geel et al., 2011).

Both extraction methods were done in duplicates and for each set an extraction blank was included. This makes a total of 32 samples. After the extraction, I performed various PCR's to optimise the results. I used different combinations of additives and also two primer sets (Table 2). One (the Z1aF, 19bR combination) is the above mentioned newly designed primer. The second (the M13F-Z1aF; M13R-19bR) is the same primer but with a M13 bacteria tail which makes it suitable for Sanger sequencing. An overview of the various PCR's is given in **Error! Reference source not found.**.
Together with Barbara Gravendeel, suitable PCR products for sequencing were selected after PCR. These samples were prepared for sequencing by cloning of the PCR product. On eight cloned colonies PCR was performed again and the products were run on a gel. The colonies containing the expected length of DNA were then sequenced.

In addition, I designed an extra set of suitable *rbcL* primers using the design program Primer3Plus. With these more targeted primers, Aline Nieman reran the previously described analysis.

TABLE 1. OVERVIEW OF SAMPLES USED FOR HISTORIC DNA ANALYSIS.

Extraction Nr	Species	Collector	Number
1	*V. grandifolia*	Léonard	1867
2	*V. crenulata*	J.J. Bos	5537
3	*V. cucullata*	Louis	834
4	*V. africana*	Peguy	128
5	*V. seretii*	Ewango	2340
6	*V. heterolopha*	Breteler	6691
7	*V.chalotii*	Strijk	244

TABLE 2. OVERVIEW OF THE PRIMERS USED IN THE HISTORIC DNA EXTRACTIONS.

Primer	target	sequence 5' - 3'	reference	note
Z1aF	rbcL	ATGTCACCACCAACAGAGACTAAAGC	Hofreiter et al 2000	157 bp incl. primer
19bR	rbcL	CTTCTTCAGGTGGAACTCCAG	Hofreiter et al 2000	157 bp incl. primer
M13F-Z1aF	rbcL	TGTAAAACGACGGCCAGTATGTCACCACC AACAGAGACTAAAGC	Hofreiter et al 2000	with M13 tail
M13R-19bR	rbcL	CAGGAAACAGCTATGACCTTCTTCAGGTG GAACTCCAG	Hofreiter et al 2000	with M13 tail

TABLE 3. OVERVIEW OF PERFORMED HISTORIC DNA PCRS. VOLUMES ARE IN ML PER SAMPLE.

PCR	Primerset	MgCl	BSA	DMSO
PCR 1	rbcL Za1F – M13 tail ; rbcL 19bR – M13 tail	0	0	0.5
PCR 2	rbclF 015 – MID 3; rbclR 015 – MID 3	0.5	0.25	0
PCR 3	rbcL Za1F – M13 tail ; rbcL 19bR – M13 tail	1	0.25	0.25
PCR 4	rbcL Za1F – M13 tail ; rbcL 19bR – M13 tail	0	0	0
PCR 5	rbcL Za1F; rbcL 19bR	1	0.25	0.25
PCR 6	rbcLF 015 - MID 2; rbcLR 015 - MID 2	1	0.25	0.25

5.2.2 REGULAR DNA METHODS

Through Marc Pignal of the herbarium in Paris, I received some fresh cuttings of living *Vanilla* plants from Cherbourgh Botanical Gardens in France (Table 4). Also, Theo Damen and Jan Wieringa collected cuttings from wild plants in Gabon. Of these plants, there was no way I could determine which species it belongs to since the plants were not flowering. With these samples, the phylogenetic tree could be expanded and hopefully, DNA barcodes for a number of *Vanilla* species can be developed.

Starting with the analysis, I extracted the DNA from the samples based on the paper of Särkinen *et al.* (Särkinen, 2012) using the three methods that where most successful in their study. First, I used a version of the CTAB protocol regularly used by the Biosystematics Chairgroup (Appendix 7). Second, I followed this CTAB protocol until the isopropanol step and then used the Promega Wizard DNA Clean-up System Kit (Corporation, n.d.) (Appendix 8). Last, I again followed the steps of the CTAB protocol until the samples were put in isopropanol and then followed the protocol of the Promega Wizard Genomic DNA Purification Kit with Vacuum Manifold (Promega Corporation, nd) (Appendix 9).

After extraction of the DNA, I performed a number of different PCR analysis for the *rbcL* region (Appendix 10). I started with a regularly used set of primers. Since the *rbcL* region is quite a long one, it is custom to use a number of sets of primers to cover the entire region. I performed PCR1, PCR2, PCR3 and PCR4 on extraction samples 2, 3 and 4 both diluted and undiluted using two primer combinations. As can be seen in Table 5, I tested various compositions of the PCR mix and a number of PCR programs which differed in annealing temperature.
Since I did not have a positive *Vanilla* control, I received some extracted *Arabidopsis* DNA from Bertha Koopmanschap from the Genetics laboratory of Wageningen University. With this material, I ran two gradient PCR's (PCR5 and PCR6) to find the right annealing temperature for the primers. PCR protocol and mixtures are the same for these two runs, but the primers differ.
As I got no results from the previous PCRs, I used the ITS1 and ITS4 primers in PCR 7, PCR 8, PCR 9 to check whether there was any DNA present in my extraction samples.
Since these were positive, we decided to use the same primers Bouetard *et al.* used in their study on my material. The compositions of the PCR mixes can be found under PCR 10, PCR 11 and PCR 12 in the table.
The PCR mixture and protocol were thus optimised for a few samples. When this was done, all samples were amplified using this protocol in PCR A and B.

TABLE 4. OVERIEW OF EXTRACTIONS.

Extraction method	Extraction Nr	Collector	Number
CTAB	SK 1	THJ Damen	487 A
	SK 2	THJ Damen	487 B
	SK 3	THJ Damen	567
	SK 4	THJ Damen	568
	SK 5	THJ Damen	569
	SK 6	THJ Damen	570
	SK 7	THJ Damen	571
	SK 8	THJ Damen	573
CTAB + Wizard Clean-up System	SK 9	THJ Damen	539
	SK 10	THJ Damen	572
	SK 11	THJ Damen	575
	SK 12	THJ Damen	576
	SK 13	THJ Damen	596
	SK 14	JJ Wieringa	7370
	SK 15	JJ Wieringa	7697
	SK 16	Cherbourgh	46
	SK 17	Cherbourgh	840
	SK 18	Cherbourgh	869
	SK 19	Cherbourgh	870
	SK 20	THJ Damen	s.n.
	SK 21	THJ Damen	54??
	SK 22		s.n.
Wizard Purification Kit	SK 23	Cherbourgh	46
	SK 24	Cherbourgh	840
	SK 25	Cherbourgh	869
	SK 26	Cherbourgh	870
	SK 27	THJ Damen	s.n.
	SK 28	THJ Damen	54??
	SK 29		s.n.

TABLE 5 OVERVIEW OF PCR'S IN THE LAB OF WAGENINGEN UNIVERSITY. PRIMERSETS: 1: 1F; 724R; 2: 636F; 1460 R; 3: ITS1; ITS4; 5: 453L/1231R.

PCR	Sample	Primerset	Buffer (µL)	dNTP (µL)	Primer forward (µL)	Primer reverse (µL)	Taq polimerase (µL)	Water (µL)	DNA (µL)	Total volume (µL)	Annealing temperature (°C)	Cycles
1	2.3.4	1	5 (10x)	10	1	1	0.5	31.5	1	50	55	30
2	2.3.4	2	5	10	1	1	0.5	31.5	1	50	55	30
3	2.3.4	1	10	10	1	1	0.5	27.5	1	50	53	30
4	2.3.4	2	10	10	1	1	0.5	27.5	1	50	58	30
5	Arabi dopsis	1	5	10	1	1	0.25	6.75	1	25	Gradient	30
6	Arabi dopsis	2	5	10	1	1	0.25	6.75	1	25	Gradient	30
7	6	3	5	10	1	1	0.25	6.75	1	25	55	30
8	6	3	10	2	1	1	0.5	34.5	1	25	60	40
9	6	3	10	2	10	10	0.5	16.5	1	50	55	32
10	6,7	4	10	2	2	2	0.5	32.5	1	50	55	32
11	6,7	5	10	2	2	2	0.5	32.5	1	50	55	30
12	6,7	5	10	2	2	2	0.5	32.5	1	50	55	30
13	6,7	5	10	2	2	2	0.5	32.5	1			
A	All	4	10	2	2	2	0.5	32.5	1	50	55	30
B	All	5	10	2	2	2	0.5	32.5	1	50	55	30

TABLE 6. PCR PROTOCOL

Step	Temperature (°C)	Time (seconds)
Denaturation	94	60
Denaturation	95	45
Primer annealing	Variable	60
Nucleotide extension	72	120
Cycles	Variable	
Tidying	72	420
End	10	Forever

After PCR amplification, I purified the PCR samples with the GeneJET PCR Purification Kit. I adjusted the protocol slightly in order to obtain the quantity of DNA necessary for sequencing at GATC Konstanz. Instead of solving the precipitated DNA in 50 µL of solution buffer, I used only 10 µL. Because the yield was so low, I then let the solution buffer completely evaporate. Then, I diluted the precipitated DNA in 5µL of MQ water. To this solution, I then added 5 µL of primer with a concentration of 5µL/µL (Appendix 11).
Hereafter, I send the PCR products to GATC Konstanz to be sequenced. We had no experience with this sequencing company and it was the question whether the PCR products would generate a usable result. Therefore, I first send four PCR products of the first primer pair (spanning the first region of the gene) in combination with their forward primer to the company for testing. Little variation was found in this region, so we decided to sequence the other region of the gene. For the second primer pair, we sequenced all 22 leaf samples with the forward primer.

After sequencing, I checked the tracers in CodonCodeAligner (Corp., n.d.). Now and then it was necessary to adjust the assigned nucleotides because of multiple peaks on one codon position.

5.2.3 General phylogenetic analysis

I used two quite different methods to generate sequence data as are described above. However the analysis done with these sequences are rather similar.

The sequences were added to the sequence alignment compiled by Bouetard *et al*. I obtained from NCBI GenBank (Appendix 12). To this alignment, I added additional sequences of other relevant species. Examples of this are sequences from the Apostasioideae, the basal sister family of the Vanilloideae within the Orchidaceae, which has not been implemented yet in the phylogenetic tree to obtain a more proper rooting. Also sequences of the Magnoliids were included since this clade is the basal sister of the Asparagales, to which the Orchidaceae belongs.

I assembled these sequences in Mesquite (Maddison & Maddison, n.d.) and aligned them by hand. Then, I assessed phylogenetic relationships between the sequences using RAxML-HPC2 on XSEDE via the CIPRES Science Gateway (Miller, Pfeiffer, & Schwartz, 2010).
Since I have such different types of data, I made a number of alignments consisting of different combinations of data. First, I made a basis alignment with all the data from Genbank (consisting mainly of the Bouetard accessions). Second, I made a historic DNA alignment, consisting of the basis alignment plus the historic DNA sequence data. Third, a regular DNA alignment, with the basis alignment extended with the sequence data obtained using the regular DNA extraction methods. Last, I implemented all available sequences in one overarching fourth alignment.

5.3 RESULTS

With both de historic DNA and regular DNA extraction methods, I was able to generate DNA sequences from various herbarium and leaf samples of *Vanilla* specimens.

Using the aDNA methods, we were able to generate sequences from two samples: nr 4 and nr 7. With the additional primers I designed, a *rbcL* sequence for an additional part of herbarium sample number 7 was generated. These collections were previously identified as *V. africana* and *V. chalotii* respectively. When blasted in Genbank, both sequences are labelled similar to a number of orchid genera, including *Vanilla.*
With the regular DNA extraction and sequence methods, the entire *rbcL* region of two leaf samples was sequenced. For 21 other leaf samples, the second half (primer pair 5) and for one the first half (primer pair 4) of this gene was sequenced. All of these turned out to be similar to other *Vanilla* sequences when blasted in Genbank.

As described in the Materials and Methods of this chapter, I made four different alignments using combinations of these sequences which all produced a phylogenetic tree. The overarching phylogenetic tree produced by the fourth alignment is shown in Figure 19. The phylogenetic inferences of the other alignments can be found in Appendix 13.

The first thing that stands out from Figure 19, is that the American clade is not monophyletic. Both *V. inodora* and *edwallii* are (South) American species, ranging from Mexico to Central America and from Brazil to Argentina respectively (Govaerts & Campacci, n.d.). But they are well separated from the rest of the American species (purple line).
The split between the American (purple line) and African (green line) clades is well supported with a node support of 98.

Within the African clade (Figure 20), we find a polytomy of splits with low support values. When looking at the phylogenetic tree of the basis alignment in Appendix 13, it can be found that originally this clade was slightly better resolved.
The lines coloured to indicate *V. imperialis* (red) and *V. africana* (blue) mostly group together, separated from each other. The exception being the sequence for *V. africana* obtained with aDNA methods (Figure 20, blue arrow).
Within the *imperialis* clade, we find the sequences of *V. polylepis*, which is part of the *imperialis* informal group as described by Soto Arenas and Cribb (2010). In addition, one sequence obtained with regular extraction methods (*Cherbourgh-869*, red arrow) is nested within this clade.
Within the *africana* clade, we find that there is a close relationship between *africana* (and related species) and the Caribbean species (*V. albida* and *V. aphylla*). Also, a quite a lot of the sequences obtained with the regular extraction methods cluster in this clade.

The placement of the historic DNA sequences is intriguing. One of the sequences (V_africana, blue arrrow) does fall within the African polytomy, but is not directly located with the other *africana* accessions. This is the same in the historic DNA phylogenetic tree (Appendix 13).
The other historic DNA sequence (V_chalotii, grey arrow), also falls within the African polytomy as is expected since it is a West African clade.

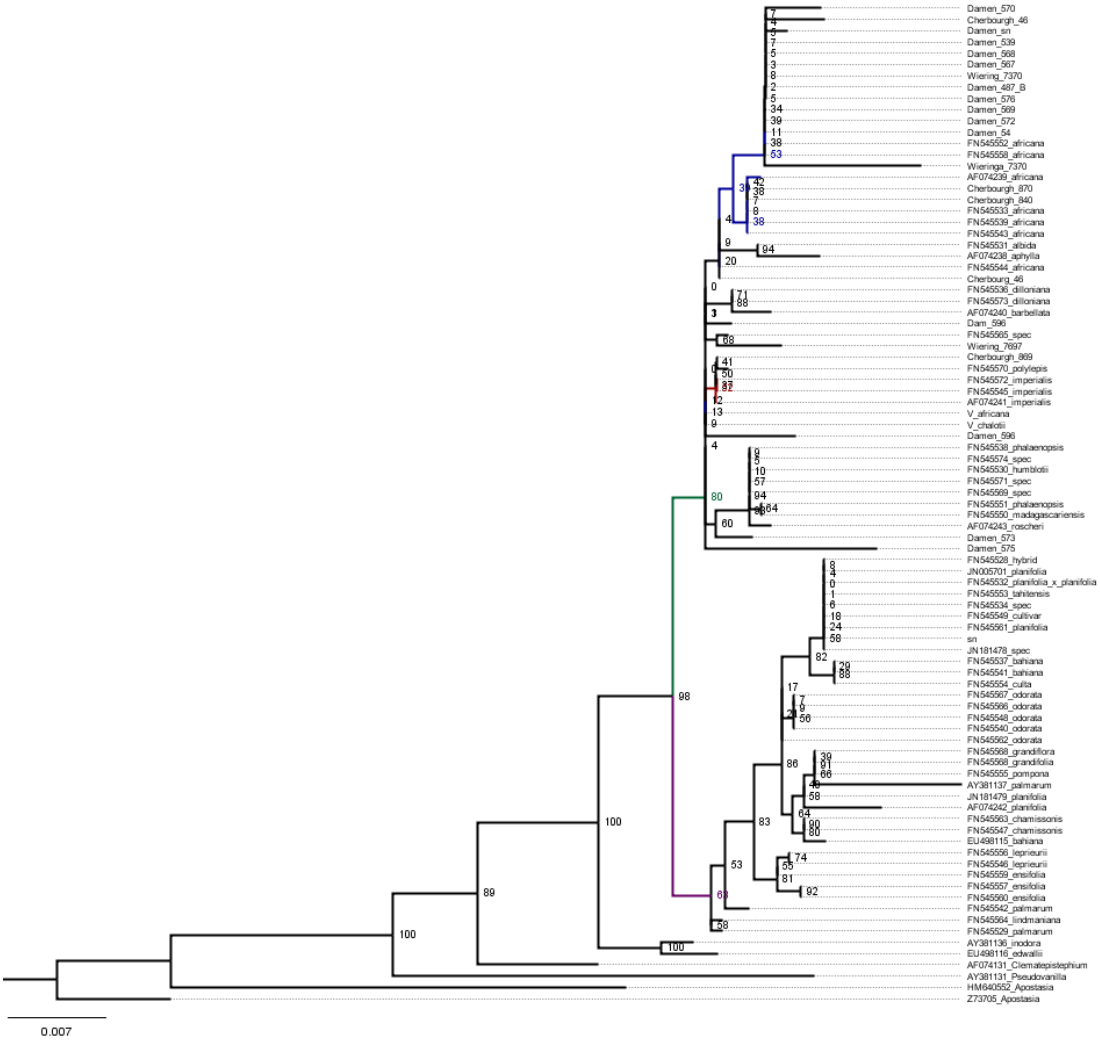

FIGURE 19. RAXML ANALYSIS OF 'OVERARCHING ALIGNMENT'. NUMBERS INDICATE BOOTSTRAP SUPPORT.

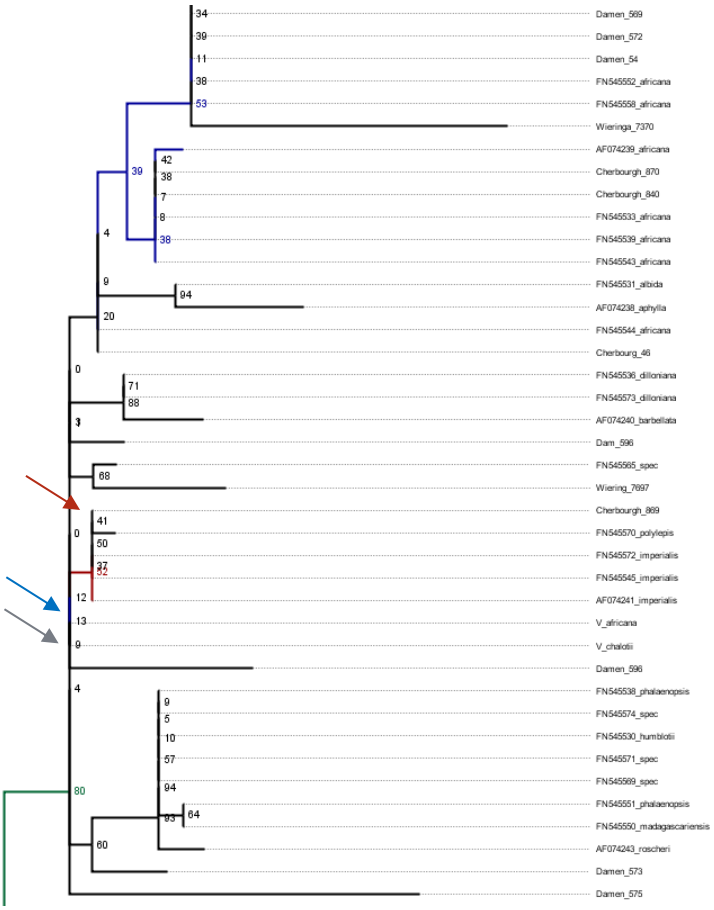

FIGURE 20. AFRICAN CLADE OF RAXML ANALYSIS ON 'OVERARCHING ALIGNMENT'. NUMBERS INDICATE BOOTSTRAP VALUES.

5.4 Discussion and Conclusion

The question addressed in this part of the research (Can (part of) the phylogenetic hypothesis of Bouetard *et al.* (2010) be complemented and optimised with additional *Vanilla* species, especially those from the *V. imperialis* & *africana* groups as described by Soto Arenas & Cribb (2010)?) is a bit arbitrary. Of course the phylogenetic tree can be complemented and optimised. But the goal (set by the underlying hypotheses) is quite plain: rather specific parts of the *Vanilla* variation are missing in this phylogenetic tree and a lot remains unclear. This has to be cleared up.
Also, it would be very handy to have a set of model sequences that each represents a different species. In this way, the species of not flowering *Vanilla* plants can be identified.

The historic DNA research was therefore set up to fill in the gaps of missing species in this phylogenetic tree. Very specific species were attempted to be added to the phylogenetic tree of Bouetard *et al.* (2010). The only way this is possible is by taking leaf samples from specimens that were identified to be those particular species based on morphology.

We were able to successfully extract, amplify and clone five out of seven selected leaf samples using aDNA methods, but due to DNA degradation three of these were contaminated with *Solanum lycopersicon* and bacterial tissue. So only two leaf samples generated a usable sequence. However, since the target region of the primer is only around 120 bp, this sequences were rather short. Also, they did not contain a lot of usable information since they were placed at the beginning of the *rbcL* gene, even just before the majority of sequences from GenBank started. Therefore, I designed an additional primer for a more variable part of the *rbcL* gene within the *Vanilla* alignment. In total, three sequences for two leaf samples were obtained in this way. One for *V. africana* (with the first primer) and two for *V. chalotii* (with both first and second primer).

To answer the first hypothesis (The *V. chalotii* group will cluster in an intermediate clade between African and Caribbean species), the sequence of the herbarium specimen of *V. chalotii* is important. As presented in the Results of this chapter, the hDNA sequence I obtained for *V. chalotii* indeed clusters within the African clade. But unfortunately not near the *africana* clade and the Caribbean species *V. dilloniana* and *V. barbellata*. Based on these results, I should thus reject this hypothesis.
However, the sequence data available for this species is still rather little, resulting in a weak node support (9) and making the placement in the phylogenetic tree dubious. I believe no sound conclusions can be based on these results and therefore not accept nor reject the first hypothesis. To be able to accept it, a strong supported sequence of *V. chalotii* or related relevant species should cluster within the *africana* clade, parting it from the Caribbean species. Now that is shown that aDNA extraction methods are possible for *Vanilla*, more primers, both for other genes and other regions of the *rbcL* gene, can be designed and the data can help form a better underpinned picture.

The second hypothesis states that 'The *V. francoisii* group will be closely related to the *V. madagascariensis* group, since both are Indian Ocean based groups'. Unfortunately, no species from the informal *V. francoisii* group could be added at all to the phylogenetic tree because extraction with aDNA methods did not work in this case. I therefore find also the second hypothesis cannot be judged.

In order to judge the third hypothesis ('The informal *V. imperialis* and *africana* groups are monophyletic groups) I included all relevant species that are missing from the phylogenetic tree of Bouetard *et al* (2010) in the historic DNA research. Unfortunately, none of these herbarium leaf samples generated a DNA sequence. Still, in the phylogenetic inference of the alignment shown in Appendix 13, the two groups both remain monophyletic. Based on this, one could accept the hypothesis. However, I again feel I cannot make a proper judgement, since the hypothesis was formulated in the spirit of the historic DNA research. Thus whether or not the informal

groups would remain monophyletic after incorporation of the missing species. This has not been answered since not all species of the groups are included in the phylogenetic tree.
In addition, I would find it dangerous to make a decision based on the phylogenetic tree of Figure 19. It gives a good indication, but overall is weakly supported and based on only one gene. Especially for the *africana* group, since the historic DNA sequence for *V. africana* (V_africana) does not cluster with the other *africana* accessions. But even more than for the *V. chalotii* sequence, it has to be remembered that there is really quite little information available in this sequence.

There are a number of general differences between my phylogenetic tree and the one of Bouetard *et al* (2010). In contrast to the phylogenetic tree of Bouetard *et al.* (2010), the American clade in the phylogenetic tree pictured in Figure 19 is not monophyletic. The species responsible for this (*V. edwallii* and *V. inodora*) do not occur in the phylogenetic tree of Bouetard *et al.*(2010). Perhaps they overlooked them when assembling their alignment of sequences from GenBank.
Why these two species stand out so clearly is a mystery to me since I did not study their morphology in relation to the other American based species. However, when checked in the Mesquite alignment, I found there are a number of codons they both have in common with the species belonging to the outgroup that differ from the *Vanilla* sequences.
In addition, my phylogenetic tree does not contain accessions of species from the informal Membranaceous group. In their paper, Bouetard *et al.* (2010) indicate they found a sequence for *V. mexicana* in GenBank and that the accession number should be listed in the attached table. But the species does not occur in the table at all, and when searched for in GenBank, no accessions from this species could be found.

How it is possible that three out of five samples were contaminated is quite strange, since we took all necessary precautions that are customary in an Ancient DNA lab. Every morning, all surfaces were cleaned with a chlorine solution and all material was put in a UV chamber to destroy any stray DNA scraps. I am positive neither Anile Nieman nor myself ate tomato during the extractions, so the contamination could not come about that way.
As it thus turns out, sequencing orchid DNA from herbarium specimens using historic DNA methods is indeed possible. More time then was available is only needed for the final fine tuning of the methods to have it work on the oddities of *Vanilla* DNA. Then, extraction and sequencing of DNA from the particular species that are part of one's research should pose no great problems. Also, in the future, more herbarium specimens may be sampled using the newly designed primer.

The second extraction path turned out to be more laborious than expected as well. It took a lot of time to successfully amplify the leaf material that was available using PCR because of numerous difficulties in the lab. A number of sequences are successfully added to the alignment, but due to limited time not the whole *rbcL* region of all silica samples could be sequenced.
Even though a limited amount of data is thus available per sequence, all of these samples do group within the African/Asian clade. This indicates that the region that is now sequenced indeed can be used as an indicator for general relationships. For sterile specimens, at least their morphological group can be established.

6. POLLINATION BIOLOGY IN *VANILLA*

Pollination is often considered a great driver for speciation since reproductive isolation of plants as a result of different pollinator syndromes causes reproductive isolation (Kiester, Lande, & Schemske, 1984; Paulus & Gack, 1990; Peakall et al., 2010). Flowers of all families are wonderfully adapted to facilitate the visitation of the pollinators that are so important for their reproduction. There are many beautiful examples of how far this relation can go. Also, a large number of species within the Orchidaceae uses (food)deception to increase chances of pollination (Caradonna & Ackerman, 2012; Pansarin et al., 2012).

In *Vanilla*, not much is known about pollination mechanisms or pollinators (Pansarin, 2013; Van Dam, 2010). Even though *V. planifolia* is the most important commercial species, not much research has been done into its pollination mechanisms in the wild.
The sister tribe Pogoniae, however, has been studied more. An extensive study by Pansarin *et al.* (2010) shows that species of the tribe are mainly pollinated by nectar seeking bees. But also pollination by hummingbirds and self-pollination were found (Pansarin, 2012).
The studies that have been done on *Vanilla* pollination concentrate on species inhabiting Middle and South America. These studies give a quite complex image of the pollination of *Vanilla*. Some species depend on pollinators to facilitate their reproduction, while others appear to be completely self-pollinating.
In the literature of the past 40 years, three mechanisms for auto-pollination are described for *Vanilla* (Van Dam et al., 2010). The first, called 'stigmatic leak', is made possible by cleistogamy. In cleistogamy, the flower doesn't open as far as it could and thus self-fertilisation is promoted (Caradonna & Ackerman, 2012). An example of this is shown in Figure 31 for *V. bicolor*. In this particular case, a fluid from the stigma that induces germination is released by the stigma. Another way of self-fertilisation is when the rostellum, located between the anther and stigma to prevent self-fertilisation, is underdeveloped or shrunken by dehydration. The third and last mode is trough agamospermy, where seeds are formed asexually (Botany online). For *V. bicolor* Van Dam *et al.* (2010) found stigmatic leak to be the primary way of auto-fertilisation. It would be quite interesting to investigate this for other species as well.

FIGURE 21. CLEISTOGAMOUS STATE SHOWN BY *VANILLA BICOLOR* (AFTER VAN DAM *ET AL.*, 2010).

In the light of my morphological research I would have liked to be able to relate the results to possible differences in pollination systems and pollinators. But since no pollination related research has been done into the wild species of Continental Africa, it will be a lot of guesswork. However, although pollinator species are not known, I can make some general comments on the pollination of both the *imperialis* as the *africana* group.

The flowers of the *imperialis* group show general resemblances with those of *V. bicolor* and *V. planifolia*. Both have the same tube shape formed by the fusion of sepals/petals, lip and column as the flowers of the *imperialis* group. Therefore, one might argue that there could be a resemblance in pollination. Perhaps not in pollinators, since *imperialis* occurs solely in Continental Africa and *V. bicolor* and its relatives only reside in America. But perhaps then in the way pollination occurs. Unfortunately, this also remains speculation since the species with flowers that resemble those of *imperialis* show great variety in their pollination mechanisms. As described above, some are quite unsuccessfully pollinated by a range of pollinators. Others are quite successful self-pollinators, but differ in the way they self-pollinate. Of the above described methods, the second doesn't seem likely, because I did not encounter any flowers with a shrivelled rostellum. I also did not come upon any flowers that were more closed than others, but this is hard to determine since I boiled the flowers and then prepared them. Also the flowers still attached to the plant on the herbarium sheet have lost their original form due to the drying process. Both the stigmatic leak and agamospermy methods I thus did not see, but it is possible *imperialis* is fertilised by one of them.

For the flowers of the *africana* group, I find it rather difficult to make any comments on pollination. I suspect some insect species are involved in the pollination rather than pollination occurring through self-pollination. This because the flowers are much more open in their morphology. The sepals and petals, lip and column are still fused to form a tube, but this tube is much smaller than the one formed by *imperialis* relative to the size of the flowers. Also, in a number of cases the tube itself is quite open at the sides (Figure 22). This makes self-pollination by 'stigmatic leak' quite difficult. Also, I again did not find any examples of shrivelled rostella. Therefore, of the pollination of *africana* as a group I cannot say anything with certainty.

Within the *africana* group, I can say that probably there are differences in pollinators. The first morphological group described in Chapter 4 has a distinctly different lip shape compared to the other groups. The lip is the landing platform for a pollinator, so a difference in shape strongly indicates a difference in pollinator. The lip of *V. heterolopha* is that much broader and larger, and the flower as a whole is much opener that it indicates a larger pollinator than is suitable for *africana*. Within the species *africana*, the diversity in flower morphology is so great that I cannot image the whole species is pollinated by just one and the same pollinator

FIGURE 22. EXAMPLE OF FLOWER WITH OPEN SIDES IN THE AFRICANA GROUP.

7. LINKING MORPHOLOGY AND DNA

The aim of this study was to link the fields of morphological and phylogenetic taxonomic research. It was already a precarious project to begin with, and unfortunately this ultimate goal is not met. Because of difficulties resulting in unfinished research in both the morphological as the phylogenetic fields, I cannot make the final step to link them. This does not mean that the project failed. Both fields are updated and a foundation for future research is laid.
Especially with the DNA based phylogenetic research, there were some unexpected issues. Beforehand, we already knew the historic DNA track would be difficult. But because of the experience with other orchid material in the lab, there was reasonable hope this kind of analysis would work on *Vanilla* as well. This indeed was the case, but as indicated in the Conclusion and Discussion of the corresponding chapter, the methods have to be adjusted a bit to the material under research.

For further development of the still remaining problem on the phylogenetic tree filled with gaps from Bouetard *et al.* (2010), my original back-up plan would still be a good option for future research. In this plan, the phylogenies of Bouetard *et al.* (2010, based on the chloroplast gene regions *rbcL, psaB, psbB and psbC*) would be linked to the 5.8S rDNA nuclear ribosomal ITS phylogenetic tree of Soto Arenas & Dressler (2010). They formed a phylogenetic tree solely based on this region for Mexican and Caribbean species. With the use of the combined samples of Bouetard *et al* and Soto Arenas & Dressler, a phylogenetic tree can then be made based on these five gene regions. This phylogenetic tree would then be well supported because it is based on both nuclear and chloroplast DNA. Also species from Asia can then be taken into the analysis to form a complete picture of the variation within the genus.
Possible hypotheses for this research would be:

A. When the nrDNA phylogenetic tree of Soto Arenas & Dressler (2010) is complemented with African samples, it will resemble the cpDNA phylogenetic tree of Bouetard *et al.* (2010).

B. The African *V. africana* and *imperialis* groups will cluster near *V. barbellata* from the Caribbean.

Much the same procedure would have to be followed as was done in this research. Leaf samples of all involved species would have to be gathered for extraction, PCR amplification and sequencing. This would be quite a challenge.
It would be important to obtain samples from *V. barbellata* since it is the only relevant species linking the two already existing phylogenies.

In the future, another option for further research would be to investigate the actual pollination process of *Vanilla* species. From a commercial point of view, it might be interesting to look into the pollination of *V. planifolia* since hand pollination, as is common practise in *Vanilla* production at the moment, is quite labour-intensive. All plants are thus effectively fertilised with their own pollen. Genetic diversity is thus more and more limited, leaving only those plants that produce the most *Vanilla* pods.

Also, since the shape of the lip seems to be quite a fascinating character dividing *V. heterolopha* and the rest of the *africana* group, it would be interesting to investigate whether this could be pollinator driven speciation. This would pose quite some problems. Finding the species at the flowering time is in itself quite a challenge as the inflorescences are mainly situated in the canopy of the forest. When successful, a possible pollinator would somehow have to be recorded.

Basically, a whole lot of opportunities are still to be explored!

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APPENDIX

1. HERBARIA ACCESSIONS

Below, I listed the accessions of all the herbaria specimen I used for this thesis (K = KEW Royal Botanical Gardens, London, Great Britain; BM = British Museum, London, Great Britain; P = Herbarium of Paris, Paris, France; BR = Herbarium of Brussels, Brussels, Belgium; WAG = Herbarium Vadense, Wageningen, The Netherlands)

Collector	Number	Species (as determined by previous researchers)	Herbarium
Ariwado	648	crenulata	WAG
Bequaert	1469		
Bequaert	1469		
Bequaert	2108		
Bequaert	2108		
Bequaert	2111		
Blickenstaff	92		
Bos	3530		
Bos	4195		
Bos	4195		
Bos	5537		
Breteler	6691		
Breteler	6691		
Daniels	85		
F de Carvalho	2896		
Fakih	169		
Fakih	169		
de Giorgi	1715		
Jongkind	5038		
Lebrun	1948		
Lebrun	1948		
Lebrun	1948		
Lebrun	2493		
Lebrun	2493		
Lebrun	3241		
Lebrun	3241		
Lebrun	3241		
Lebrun	3241		
Leeuwenberg	3054		
Leeuwenberg	3054		
Leeuwenberg	5465		
Leeuwenberg	5465		
Leeuwenberg	5465		

Louis, Breteler & de Bruijn		
Louis	8577	
Peguy	128	
Simons & Westerduijn		
Versteegh & den Outer		
de Wilde	576	
de Wilde	576	
de Wilde	843	
Tisserant	863	
Tisserant	863	crenulata
Tisserant	s.n.	crenulata
?	s.n.	africana
Bonardi	s.n.	crenulata
Chevalier	17504	africana - africana
Aubreville	s.n.	crenulata
Bonardi	s.n.	crenulata
Chevalier	22383	africana - africana
Chevalier	34171	crenulata
Thollon	160	crenulata
Le Testu	1413	crenulata
Klaine	776	crenulata
Le Testu	7494 bis	heterolopha
Letouzey	9336 bis	crenulata
Adam	16656	africana
Linder	46	crenulata
Chevalier	21110	crenulata
Chevalier	19854	africana - africana
		africana
Leeuwenberg	5465	ramosa
Zenker	525	cucullata
Bos, JJ	3251	crenulata
Bos, JJ	5537	crenulata
Bos, JJ	4765	crenulata
Tisserant	2409	ramosa
Farron	7088	ramosa
Trilles	21	ramosa
Perez-Vera	169	africana
Pignal	2526	africana
Tisserant	2409	ramosa
Bos, JJ	4195	crenulata
Thollon	322	ramosa

Klaine	189	ramosa
Le Testu	9015	ramosa
Le Testu	7723	ramosa
De Giorgi	1715	laurentiana = ramosa
Blickenstaff	92	crenula
Chevalier	17504	africana africana
Chevalier	22383	crenata
Breteler	6691	africana
Leeuwenberg	5465	ramosa
Lebrun	3241	ramosa
Lebrun	2493	ramosa
Faulkner, HG	3121	zanzibarica = ramosa
Williams, RO	64	
Segerback	1537	
Perez-Vera	561	
Bowling	36605	
Greenway, PJ	7043	zanzibarica = ramosa
Greenway	7043	zanzibarica = ramosa
Moreau, WM	408	zanzibarica = ramosa
Greenway	2606	zanzibarica = ramosa
Baldwin, JT Jr	10661A	crenulata
Leeuwenberg	3054	crenulata
Tessmann, G	224	ramosa
Louis, Breteler & de Bruijn	834	crenulata
?	859	africana
Deighton, FC	6151	crenulata
Andol, FH	5257	ramosa
Le Testu	7494	heterolopha
Bates, GL	1117	ramosa
Miles, AC	sn	crenulata
Bunting, RH	3	crenulata
Le Testu		heterolopha
Le Testu	1413	crenulata
Le Testu	2409	ramosa
Le Testu	7723	ramosa
Le Testu	863	ramosa
Le Testu	9015	ramosa
Le Testu	7494	heterolopha
Linder, DH	46	crenulata
Baldwin, JT Jr	11116	crenulata
Baldwin, JT Jr	10966	crenulata
Barker, AJD	1265	crenulata

Leeuwenberg	3054	crenulata	
Deighton, FC	2963	crenulata	
Johnson, WH	230	crenulata	
Bowling	38137	crenulata	
?	sn	crenulata	
Wistwood, D	112	crenulata	
Irvine, JR	1186	crenulata	
Wigne	3062	crenulata	
Baldwin, JT Jr	10661a	crenulata	
Deighton, FC	2496	crenulata	
?	798	africana	
Kennedy, JD	2081	ramosa	
Talbot, PA	sn	ramosa	
Rowland		ramosa	
Bowling, JC	GC36647	ramosa	
Lyne			
?	?	crenulata	BR
?	525	africana - cucullata	MO
Achten, L	521	seretii	BR
Bates, GL	1117	africana	BR
Bequaert	2671	seretii	BR
Bequaert	6733	crenulata	BR
Bos, JJ	3251	africana - africana	WAG
Bos, JJ	5992	crenulata	WAG
Bos, JJ	4310	crenulata	WAG
Breteler, FJ	14547	africana	WAG
Carvalho	6262	chalotii	MA
Claessens, J	638	africana	BR
Darbyshire, I	283	ramosa	WAG
de Wilde, JJFE	576	crenulata	WAG
Dewulf, A	502	africana - ramosa	BR
Evrard, C	551	ramosa	WAG
Evrard, C	551	africana - ramosa	BR
Fakih, SA	169	ramosa	LISC
Germain, R	4520	crenulata	BR
Germain, R	2374	africana	BR
Jongkind	6873	africana	WAG
Kennedy, JD	2081	africana	BR
Laurent	1019	crenulata	BR
Laurent, Em & M	5538	africana	BR
Laurent, Em & M	?	crenulata	BR
Le Testu	863	africana - ramosa	BR

Le Testu	7723	africana - ramosa	BR
Lebrun	2493	ramosa	WAG
Louis, AM	2236	heterolopha	WAG
Louis, AM; Bretelerr, FJ; de Bruijn, J	834	africana - cucullata	WAG
Louis, J	796	laurentiana	MA
Louis, J	4502	crenulata	BR
Louis, J	7396	africana - africana	BR
Louis, J	14189	africana - africana	BR
Louis, J	8577	africana - ramosa	BR
Louis, J	13613	crenulata	BR
Luke	3047	ramosa	MO
Morton, JK	GC6553	crenulata	WAG
Oldeman, RAA	575	africana	WAG
Onana, JM	2822	ramosa	WAG
Onana, JM	2823	acuminata	WAG
Pittery	68	crenulata	BR
Pittery	638	africana - ramosa	BR
Seret, F	864	africana - ramosa	BR
Seret, J	737	crenulata	BR
Seret, J	285	africana	BR
Shu Neba, G	X6469	africana	WAG
Shu Neba, G	X 4817	africana	WAG
Shu Neba, G	4814	africana	WAG
Thomas, DW	5922	africana - ramosa	BR
Thomas, DW	5922	africana - ramosa	WAG
Thomas, DW	5922	africana - ramosa	MO
Thomas, DW	6895	africana - ramosa	MO
Vermoesen, F	1869	-	BR
Zenker	2457	cucullata	WAG
Gerard	5206	grandifolia - var lujae	BR
Gerard	5206		
Jacques-Felix	4727	ochyrae	WAG
Jacques-Félix	4727	ochyrae	WAG
Lebrun	446	imperialis	BR
J-PA Lebrun	446		
J-PA Lebrun	446		
J-PA Lebrun	446		
J-PA Lebrun	446		
Zenker, Coll Permaeten	391	imperialis	BR
Zenker, Coll Permaeten	391	imperialis	BR
Gerard	5206		
Seret	670		

Seret	670		
Gerard	5206		
Endengle	113	imperialis	
Prevort	s.n.		
Jacques - Felix	4727		
Bates, GL	1523		
Bates, GL	1523		
Dummer, R	2672a		
Vigne,	2412		
Hall, B	GC5391		
Wistwood, D	113		
Greenway	2928		
?	1045		
Moreau	429		
J.B. Hall	GC35391		
?	?	grandifolia	BR
?	244	imperialis	BR
?	?	imperialis	BR
Arends, JC	920	grandifolia - var grandifolia	WAG
Bos, JJ	5008	grandifolia	WAG
Breteler, FJ	1014	ochyrae	WAG
Breteler, FJ	6826	grandifolia	WAG
Chase	3896	grandifolia	BR
Claessens, J	288	grandifolia - var grandifolia	BR
Ge Giorgi	1058	imperialis	BR
Gerard	5078	grandifolia - var lujae	BR
Gerard	3226	imperialis	BR
Gillet, J	200	grandifolia - var grandifolia	BR
Gillet, J	?	grandifolia - var lujae	BR
Goossen, V	2857	imperialis	BR
Goossen, V	2857	imperialis	BR
Hulstaert, RP	1079	imperialis	BR
Hulstaert, RP	1387	imperialis	BR
Katende, AB	ATBP 519	imperialis	MO
Laurent, Em & M		grandifolia - var grandifolia	BR
Laurent, Em & M	939	imperialis	BR
le Testu	2409	imperialis	BR
le Testu	6077	grandifolia	BR
le Testu	9308	grandifolia	BR
le Testu	6077	grandifolia	MO
Lebrun, J	6257	grandifolia	BR
Lejoly, J	2860	imperialis	BR

Leonard, A	1867	grandifolia - var grandifolia	BR
Leonard, A	1867	grandifolia - var grandifolia	WAG
Louis, J	16038	grandifolia	BR
Louis, J	3599	grandifolia	BR
Louis, J	10800	grandifolia	BR
Louis, J	10800	grandifolia	MO
Louis, J	3599	grandifolia	MO
Rosselet	?	grandifolia - var lujae	BR
Seret, F	670	imperialis	BR
Seret, F	652	imperialis	BR
Seret, F	170	imperialis	BR
Solheit, J	49	imperialis	BR
Wieringa, JJ	4447	grandifolia	WAG
Liogier	15640	barbellata	
?	?	barbellata	NY
Leon	11447	dilloniana	NY
Stimson, R	1276	dilloniana	NY
Axelrod, F & Chavez, P	4535	dilloniana	NY
Liogier, AH	14885	barbellata	NY
Nee, M	44080	claviculata	NY
Leon	6458	claviculata	NY
Mejia		poitaei	NY
Pennington, C		poitaei	NY
Ackerrman, JD	2429	poitaei	NY
Brace, LJK	2043	poitaei	NY
Britton, NL; Dutcher, BH; Brown, S	5750	poitaei	NY
Ewango, Kahindo, Nobirabo	2340	seretii	MO
Strijk, JS	244	chalotii	WAG
Armando	955	roscheri	MA
Torre, AR & Correia, MF	17120	roscheri	LISC
Watmoush, R	?	roscheri	LISC
Domagg	32343	roscheri	LISC
Tweedie, EM	sn	roscheri	MO
Hiemstra in de Koning, J	8644	roscheri	MO
Groenendijk, EMC & de Koning, J	316	roscheri	MO
de Koning, J & Hiemstra, F	8990	roscheri	MO
de Koning, J & Boane, c	8749	roscheri	MO
Nappes, D	1468	roscheri	BR
Jongkind, CCH	3659	madagascariensis	WAG
Robertson, SA	2546	phalaenopsis	WAG
Leeuwenberg, 10785		roscheri	WAG
Viegas, J	12	planifolia	LISC

van der Meer, J	100	planifolia	WAG
?	298	planifolia	WAG
?	?	planifolia	WAG
Bos	1515	planifolia	WAG
Lebrun	1459	bampsiana	WAG
?	297	planifolia	WAG
Mestdagh	?	spec.	BR
?	1001	spec.	MA
?	G22	spec.	MA
?	148	spec.	MA
Barros Machado, A	178	spec.	LISC
Leeuwenberg, AJM	5465	spec.	LISC
Chase, NC	33396	spec.	LISC
Viegas, J	4544	spec.	LISC
Santo, E	4544	spec.	LISC
Seret, F	39	spec.	BR
Seret, F	39	spec.	BR
Seret, F	651	spec.	BR
Wagemans, J	1599	spec.	BR
Wagemans, J	1600	spec.	BR
?	?	spec.	BR
?	?	spec.	BR
Gillet, J	?	spec.	BR
Gille, P		spec.	BR
Gille, P	245	spec.	BR
Vermoesen, F	?	spec.	BR
Wellens, F	476	spec.	BR
Leeuwenberg, AJM	5465	spec.	BR
Harris, DJ; Michael, J	51	spec.	MO
van Valkenburg, JLCH	2861	spec.	MO
van de Laan	1356	spec.	WAG
Laan	1001	spec.	WAG

2. R Script Principal Component Analysis for the africana group

```
PCA2 <- read.table("H:\\Afstudeervak 1\\Data\\africanagemiddeld.csv",header=TRUE,sep=",")

PCA2 [,-c(1)]-> africanapcadata

rownames(africanapcadata) <-
c("1","2","3","4","5","6","7","8","9","10","11","12","13","14","15","16","17","18","19","20","21","22","23","24
","25","26","27","28","29","30","31","32","33","34","35","36","37","38","39","40","41","42","43","44","45","4
6","47","48","49","50","51","52","53","54","55","56","57","58","59","60")

africanapca <- prcomp(na.omit(africanapcadata),scale=TRUE)

summary(africanapca)

biplot(africanapca,cex=c(0.7,1.1))

print(prcomp(na.omit(africanapcadata),scale=TRUE))
```

3. HERBARIUM SPECIMENS PER MORPHOLOGICAL GROUP

Group	Collector	Number	Herbarium	Flower number
1	Breteler	6691	WAG	14
	Breteler	6691	WAG	15
2	De Giorgi	1715	WAG	20
	Ariwaodo	648	WAG	2
	Lebrun	2493	BR	26
	Bos	4195	WAG	11
	Bequaert	1469		4
	Lebrun	1948	BR	24
	Lebrun	3241	WAG	29
	Lebrun	3241	WAG	30
	Bequaert	2108	BR	7
	Bos	3530	WAG	10
3	Ariwaodo	648	WAG	3
	Lebrun	3241	BR	27
	Lebrun	1948	BR	23
	Leeuwenberg	3054	WAG	32
	Lebrun	2493	WAG	25
	Simons&W	338	WAG	39
	De Wilde	576	WAG	41
	Leeuwenberg	5465	MO	33
4	F. de Carvalho	2896	MA	17
	Bequaert	2108		6
	Jongkind	5038	WAG	21
	Bos	4195	WAG	12
5	Leeuwenberg	3054	WAG	31
	Fakih	169	MO	19
	Bequaert	1469	BR	5
	Leeuwenberg	5465	WAG	35
	Leeuwenberg	5465	WAG	34
	Versteegh & den Ouden	227	WAG	40

4. SUMMARY AND PRINT PCA IN R

IMPERIALIS GROUP

	PC1	PC2	PC3	PC4	PC5	PC6
Standard deviation	1.9080	1.2936	1.0799	0.71114	0.11946	3.637e-16
Proportion of Variance	0.5201	0.2391	0.1666	0.07225	0.00204	0.000e+00
Cumulative Proportion	0.5201	0.7591	0.9257	0.99796	1.00000	1.000e+00

	PC1	PC2	PC3	PC4	PC5	PC6
total_length	-0.4329647	0.2824929	-0.39427196	-0.07181637	0.0977742	-0.63075342
colum_length	-0.4362589	-0.2533108	-0.40413598	0.07247727	-0.6822856	0.33350882
colum_width	-0.2750196	-0.5652310	-0.08482407	0.59509925	0.4232408	-0.14673254
lip_length	-0.4057760	0.4329275	-0.24440075	-0.17159219	0.4112158	0.40027698
lip_width	-0.1533585	0.5567876	0.36039618	0.69373443	-0.2297592	0.03679212
angle_left	-0.4382548	-0.1608629	0.45537991	-0.17087932	0.2485816	0.37783257
angle_rigth	-0.4078526	-0.1150207	0.52634648	-0.30908778	-0.2492252	-0.40627426

AFRICANA WITH FANS

	PC1	PC2	PC3	PC4	PC5	PC6	PC7	PC8	PC9
Standard deviation	1.8781	1.2665	1.1215	0.86956	0.75222	0.7016	0.68667	0.44680	0.35444
Proportion of Variance	0.3919	0.1782	0.1398	0.08402	0.06287	0.0547	0.05239	0.02218	0.01396
Cumulative Proportion	0.3919	0.5701	0.7099	0.79390	0.85677	0.9115	0.96386	0.98604	1.00000

	PC1	PC2	PC3	PC4	PC5	PC6	PC7	PC8	PC9
LCT	0.4808846	-0.02550818	-0.18908627	0.2019406	-0.06544758	-0.039870114	0.09356362	0.45218533	-0.68736232
LC	0.4016701	0.07250411	-0.26809257	0.1720680	0.01513575	-0.750354630	-0.08338800	-0.34520261	0.20626119
LLV	0.3766434	-0.06639792	-0.34359370	0.2839644	-0.41740510	0.539404927	-0.22093251	-0.08633509	0.36549836
WLT	0.2985377	0.49153989	0.17208246	-0.4793817	-0.14997465	-0.093780509	-0.10417105	0.50289980	0.33881790
DAF	0.3761927	0.24343362	-0.17178700	-0.3836815	0.41745687	0.336138133	0.36901877	-0.43350889	-0.10551287
A3	-0.2779275	0.19026382	-0.59612249	0.2315367	0.49411748	0.022872942	0.08438742	0.39987737	0.25668327
A1	0.3023858	-0.39920102	0.38204255	0.2473096	0.19261212	-0.005960322	0.54840684	0.21950720	0.39498660
A2	-0.2147366	0.55104330	0.02521214	0.3168026	-0.44142387	-0.030149490	0.58306019	-0.11104125	-0.03444683
F	0.1418183	0.43543303	0.46177787	0.5067774	0.38521595	0.145496775	-0.37499890	-0.08749973	-0.04881058

AFRICANA WITHOUT FANS

	PC1	PC2	PC3	PC4	PC5	PC6	PC7	PC8
Standard deviation	1.8582	1.2510	0.9748	0.80274	0.71703	0.66417	0.48633	0.44245
Proportion of Variance	0.4316	0.1956	0.1188	0.08055	0.06427	0.05514	0.02956	0.02447
Cumulative Proportion	0.4316	0.6272	0.7460	0.82656	0.89082	0.94597	0.97553	1.00000

	PC1	PC2	PC3	PC4	PC5	PC6	PC7	PC8
LCT	0.4780531	-0.0224381	0.1621013	-0.2150319	0.0017319	-0.1431163	0.5770236	0.5873709
LC	0.4382562	-0.0314608	0.1061573	0.1556903	0.1684958	-0.7645296	-0.3655250	-0.1578858
LLV	0.3975973	-0.1741724	0.2018432	-0.3932224	-0.6626778	0.1718079	-0.1251189	-0.3631817
WLT	0.3382765	-0.3104869	-0.5902099	0.2020619	0.1698161	0.0947601	0.4152107	-0.4356295
DAF	0.4248587	-0.2693173	0.0118765	0.1918002	0.2645381	0.5255704	-0.5032471	0.3325243
A3	-0.1471740	-0.4961003	0.6857777	0.0661765	0.3391567	0.0638545	0.2394105	-0.2848360
A1	0.2309809	0.5621642	0.0942072	-0.4604555	0.5180855	0.2067710	0.0017333	-0.3139347
A2	-0.2266382	-0.4869215	-0.3066887	-0.6934572	0.2240675	-0.1829169	-0.1865535	0.1346646

AFRICANA WITHOUT FANS AVERAGE

	PC1	PC2	PC3	PC4	PC5	PC6	PC7	PC8
Standard deviation	1.8584	1.2859	0.9249	0.79779	0.7217	0.66507	0.49092	0.44336
Proportion of Variance	0.4317	0.2067	0.1069	0.07956	0.0651	0.05529	0.03013	0.02457
Cumulative Proportion	0.4317	0.6384	0.7453	0.82491	0.8900	0.94530	0.97543	1.00000

	PC1	PC2	PC3	PC4	PC5	PC6	PC7	PC8
LCT	0.4769624	-0.0248867633	0.2264883	-0.1633945	0.01898707	-0.1246139	0.5210864	-0.6375520
LC	0.4384353	-0.0392348863	0.0998576	0.1731619	0.10505737	-0.7709750	-0.3570576	0.1826166
LLV	0.3978388	-0.1695147546	0.2003746	-0.3964175	-0.6590915	0.1961445	-0.0553226	0.3738414
WLT	0.3351084	-0.3010081880	-0.6498408	0.1121419	0.19666293	0.0548111	0.4393534	0.3570931
DAF	0.4226523	-0.2720408535	-0.0378243	0.1835013	0.24340420	0.5145159	-0.5559743	-0.2813816
A3	-0.1325767	-0.5415108989	0.6386900	0.1738452	0.31843374	0.0762854	0.2391498	0.2943297
A1	0.2375694	0.5295669900	0.1679199	-0.4385330	0.55545395	0.1777521	0.0110661	0.3199436
A2	-0.2388664	-0.4804358800	-0.1968139	-0.7192972	0.21528991	-0.2155225	-0.1961226	-0.1572776

5. aDNA SILICA EXTRACTION PROTOCOL

1. When possible remove the surface of the sample, cut off a piece and grind it (freezermill or with mortar and pestill)
2. put 0.1 – 0.25g of that ground material (powder) into 2ml buffer

   for 10 samples (9 samples plus Extraction-blank) 50ml of the Extraction buffer:

45ml	0.5M EDTA pH 8.0	→	0.45M
1.25ml	10mg/ml PK	→	0.25mg/ml
3.75ml H_2O			

3. seal with Parafilm, rotate over night at RT in the dark
4. centrifuge at 4000rpm for 2min
5. add supernatant to 8ml L2 and add 40µl Silica, keep bone pellet in the freezer

   L2 for two samples, don't forget to prep. one extra tube! (or: make a 500-1000ml bottle):

24g	GuSCN	→	5.0M (drives DNA to the silica)
2ml	1M Tris pH 8.0	→	0.05M
18ml	H_2O		
180µl	NaCl 5M	→	0.025M

6. seal with Parafilm and rotate for 3h at RT in the dark
7. centrifuge at 4000rpm for 1min
8. discard supernatant into new labelled tubes (keep in fridge until you know everything worked o.k., then throw away)
9. add 1ml L2 to the pellet, resuspend and transfer to a fresh labelled 2ml tube
10. centrifuge for 5sec. in minicentrifuge
11. take off supernatant, resuspend in 1ml New Wash
12. centrifuge for 10sec. in minicentrifuge
13. take of supernatant (throw away), spin briefly, remove remaining liquid
14. dry at RT for 10min with open lids
15. add 50µl 1xTE, resuspend, elute at RT for 8min with closed lids
16. spin at max. speed for 1min, remove TE to a fresh tube = sample, take care to avoid any traces of silica, keep silica pellet in the fridge
17. Before use centrifuge briefly to pellet silica
18. Carry out a PCR immediately as freezing already detoriates the DNA.
19. freeze at -20°C, use within 4-5 month (usually 5µl per PCR), 1 PCR immediately

6. aDNA CTAB EXTRACTION PROTOCOL

Used chemicals

- CTAB extraction buffer
- chloroform-isoamyl alcohol (24:1)
- isopropyl alcohol (cold)
- 1x TE buffer
- RNAse
- 5M NH4 acetate
- 100% ethanol (cold)
- 76% ethanol, 10 mM NH4 acetate

Extraction procedure

Rough DNA extraction

1) Preheat a water bath at 60°C, insert a tube with sufficient CTAB extraction buffer (2 mL for each sample) with 2% (40 µL per sample) 2-mercapto-ethanol added beforehand in the fume hood. Check temperature with thermometer.
2) Put sample material in the mortar and add a little bit of sterile sand.
3) Carefully add liquid nitrogen, hold pestle in the nitrogen to cool, wait until part of the nitrogen has evaporated and grind the sample material. (work with safety glasses).
4) Put the sample in a 1,5 mL tube with a cooled spatula, close the tube and put in nitrogen. Clean grinder, pestle and spatula before grinding the next sample.
5) Carefully open the tubes and add 1 mL prepared CTAB buffer. Incubate for 1 hour at 65°C, shake every 10-15 minutes.

Removal of proteins

1) Add 450 µL chloroform-isoamyl alcohol (24:1) in fume hood, mix by inverting for 5 minutes, under fume hood.
2) Centrifuge for 10 min. at 13200rpm.
   Three phases are visible:
   - Bottom- chloroform with dissolved proteins, lipids and chlorofyl.
   - Middle- cell residue and dissolved green cell residue.
   - Top- nucleic acids and dissolved polysacharids – water phase.
3) Carefully take 800 µL of the water phase and put in a new tube.
4) Add 450 µL chloroform-isoamyl alcohol (24:1). Mix by inverting for 5 min. under fume hood.
5) Centrifuge samples for 10 min. at 13200rpm.
6) Carefully take 550 µL of the water phase and put it in a new 2 mL tube.

DNA precipitation

1) Add 550 µL cold isopropyl alcohol. Mix by carefully inverting for 5 min. at room temperature, white flakes should appear.
2) Centrifuge for 10 min. at 5000 rpm.
3) Drain the isopropyl alcohol. Place the tubes upside-down on a tissue to drain as much of the isopropyl alcohol as possible. Centrifuge open tubes for 1 min. at 5000rpm.
4) Dissolve the pellets in 300 µL TE, put at 37°C for 30-60 min. Add 3 µL RNAse, put at 37°C for 60 min.

~Possible stop~

- Keep samples in 4°C fridge overnight
- Put the samples at 37°C for 30 min. before continuing the extraction

Precipitation in high salt concentration – extra purification

1) Add 300 µL 5M NH4 acetate (final concentration 2,5M).
2) Add 1500 µL of 100% cold ethanol (2,5 volumes, 2x 750 µL) .
3) Put in -20°C freezer for a minimum of 20 min.
4) Centrifuge for 10 min. at 13200 rpm at 4°C, carefully drain ethanol.
5) Wash with 500 µL 76% ethanol, 10 mM NH4 acetate, swerve carefully and centrifuge for 10 min at 13200 rpm at 4°C, and carefully drain ethanol.
6) Repeat previous step.
7) Place the tubes upside-down on a tissue to drain remaining ethanol. Centrifuge open tubes for 1 min. at 5000rpm. Make sure all ethanol is evaporated.
8) Add 50 µL TE and put at 37°C for at least an hour to dissolve.

7. CTAB DNA ISOLATION PROTOCOL FOR PELARGONIUM

Modified for *Pelargonium* from Doyle and Doyle 1987 (Phytochem. Bull. 19:11-15).

Homogenisation of leaf material

1. Put 1-10mg Silica gel-dried leaf material in a 2ml- reaction tube.

You only need a very small fraction of a leaf for a DNA extraction. When starting DNA extraction with a new group it is useful to weigh out a sample once in order to get an idea how much leaf material represents 1-10mg.

2. Add 5 small glass beads.

You can also use 3 or more, large glass beads. This can sometimes be more efficient.

3. Put reaction tubes in an 8 -tube holder in the **electric bead mortar**.

Make sure to fix it tightly in order to prevent the samples to be launched and fly through the machine when switching it on. Also make sure to fix the lid of the machine properly.

4. Homogenize for at least **2 min at 70 rpm**.

Check whether the leaf material has turned into a fine dustlike powder. If not, continue homogenizing. If necessary the powder can be stored dry at RT.

Extracting the DNA

5. Add **1ml CTAB buffer. Mix** and incubate the reaction for **30 min at 55$_{o}$C** (water bath).

-- If necessary try gentle swirling during incubation and/or try longer (up to 60 min.) or shorter (15 min.) incubation time.

-- CTAB (Cetyltrimethylammoniumbromide) is a detergent that dissolves the nuclear membrane and allows the DNA to separate from polysaccharides, proteins and phenolic compounds by forming a complex.

-- PVP (in the isolation buffer) helps to remove phenolic compounds, tannins and quinones.

6. Add 1ml Chloroform/ Isoamylalcohol. Vortex carefully for about 1min.

Wear gloves and work in the fumehood, chloroform is volatile and toxic (check safety sheet).

7. **Spin down** at maximum speed (13.000rpm) in an Eppendorf centrifuge for 2min

Two phases will form: a lower organic phase (containing the chloroform with the dissolved proteins, lipids and chlorophyll) and a top water-containing phase, in which the DNA and polysaccharides are dissolved. In between the two phases a white green or brown layer (interphase) is formed containing cell debris and denatured proteins.

8. Transfer **supernatant** to a fresh **1.5 ml tube**. Be careful not to transfer cell debris from the interphase or any chloroform.

In case any interphase or chloroform has been transferred repeat the chloroform extraction (step 6, 7). If you have disturbed the layers while trying to remove the supernatant, centrifuge again for 2 min and carefully transfer the supernatant. Once the supernatant has been transferred pour off the chloroform waste in the plastic bottle underneath the fume hood.

9. Repeat Chloroform extraction with 1ml Chloroform/ Isoamylalcohol (as step 6,7,8).

10. **Spin down** at maximum speed (13.000rpm) in an Eppendorf centrifuge for 2 min.

11. Transfer **supernatant** to a fresh **1.5 ml tube**.

12. Add 2/3 volume of cold **Isopropanol, shake** well and place at **–20oC 30 min** to precipitate the DNA.

(It is possible to interrupt the DNA isolation at this point and leave the DNA over night at -20 oC.)

In the presence of salt, Isopropanol precipitates the DNA. Under low temperatures the solubility of the DNA is reduced. For some taxa ethanol has been demonstrated to result in improved precipitation.

13. **Spin down** at maximum speed (13.000rpm) for **10 min** to collect the DNA at the bottom of the

tube. After the centrifugation immediately decant the supernatant.

If you wait too long the DNA pellet may get loose from the bottom of the tube. In that case it is safer to spin down the tube again before removing the supernatant.

14. Add **1 ml wash buffer** to dissolve polysaccharides and remove any residues from the DNA. **Spin**

down for 5min. Decant the supernatant immediately.

After decanting remove remaining traces of liquid as much as possible as follows:

- put the tubes back in the centrifuge, making sure to put them in the same direction as in the last spin, otherwise the pellet may comes loose from the bottom of the tube due to the centrifugation

- spin down for some seconds

- **remove** all remaining **traces of Ethanol** with a 100µl pipet, taking utmost care not to remove the pellet.

15. **Air-dry** the samples by leaving the tubes open on the bench top.

The samples should be dry within 5 min if all traces of liquid had been removed completely. Otherwise, leave samples at RT until they are dry (optionally, use a Speedvac).

Removing Ethanol completely is important since remaining traces will suppress subsequent enzyme activity.

16. Resuspend the dry DNA pellets in 90 µl resuspension buffer

17. Optional: Removal of RNA (optional) and polysaccharides through additional precipitation:

add 1µl RNase with a final concentration of 1µg/µl to remove the RNA from the samples, incubate for **10 min** at room temperature

18. Add 180 µl MQ water 135 µl 7.5M NH_4Ac (2.5M) and 1ml cold 96% ETOH (2.5 Vol.), shake well

and put at –20oC for about **30 min** to precipitate the DNA.

(It is possible to interrupt the DNA isolation at this point and leave the DNA over night at -20 oC.)

In the presence of salt, Ethanol precipitates the DNA. Under low temperatures the solubility of the DNA is reduced. Polysaccharides stay dissolved because of the high salt concentration.

19. **Spin down** at maximal speed (13.000rpm) for **10 min** to collect the DNA at the bottom of the

tube. After the centrifugation immediately decant the supernatant.

If you wait too long the DNA pellet may get loose from the bottom of the tube. In that case it is safer to spin down the tube again before removing the supernatant.

20. Add **1 ml 70% Ethanol** to remove salt residues, **spin down** for 5 min. After the centrifugation

immediately decant the supernatant (see step 19)

After decanting remove remaining traces of liquid as much as possible as follows:

a. put the tubes back in the centrifuge, making sure to put them in the same direction as in the last spin, otherwise the pellet may comes loose from the bottom of the tube due to the centrifugation

b. spin down for some seconds

c. **remove** all remaining **traces of Ethanol** with a 100µl pipet, taking utmost care not to remove the pellet.

21. **Air-dry** the samples by leaving the tubes open on the bench top.

The samples should be dry within 5 min if all traces of liquid had been removed completely. If not, leave samples at RT until they are dry (optionally, use a Speedvac). Removing Ethanol completely is important since remaining traces will suppress subsequent enzyme activity.

22. **Resuspend** the dry DNA pellets in **100 l TE**

DNA is stored in the TE buffer pH8 which contains Tris and EDTA. EDTA inhibits nuclease activity by chelating of Mg^{2+}.

8. PROMEGA WIZARD PROTOCOL FOR DNA CLEAN – UP SYSTEM USING A VACUUM MANIFOLD

Multiple samples can be easily processed simultaneously using the Vac-Man Laboratory Vacuum Manifold. The Wizard DNA Clean-Up System is not suitable for use with RNA because percent recoveries are less than 50%.

Notes:

1. Thoroughly mix the Wizard DNA Clean-Up Resin before removing an aliquot. If crystals or aggregates are present, dissolve by warming the resin to 37°C for 10 minutes. The resin itself is insoluble. Cool to 25–30°C before use.

2. The binding capacity of 1ml of resin is approximately 20µg of DNA.

Materials to Be Supplied by the User

• 80% isopropanol (2-propanol, reagent grade)
• prewarmed (65–70°C) deionized water or TE buffer

The sample volume must be between 50 and 500µl. If the sample volume is less than 50µl, bring the volume up to at least 50µl with sterile water. If the sample volume is >500µl, split the sample into multiple purifications.

1. Use one Wizard® Minicolumn for each sample.

2. Attach the provided Syringe Barrel to the Luer-Lok extension of each Minicolumn. Insert the tip of the Minicolumn/Syringe Barrel assembly into the vacuum manifold.

Mix the resin before use.

3. Add 1ml of Wizard DNA Clean-Up Resin to a 1.5ml microcentrifuge tube. Add the sample (50–500µl) to the Clean-Up Resin and mix by inverting several times.

4. Pipet the resin/DNA mix into the Syringe Barrel. Apply a vacuum to draw the solution through the Minicolumn. Break the vacuum to the Minicolumn.

5. To wash the column, add 2ml of 80% isopropanol to the Syringe Barrel, and re-apply a vacuum to draw the solution through the Minicolumn.

6. Dry the resin by continuing to draw a vacuum for 30 seconds after the solution has been pulled through the column. Do not dry the resin for more than 30 seconds. Remove the Syringe Barrel and transfer the Minicolumn to a 1.5ml microcentrifuge tube. Centrifuge the Minicolumn at maximum speed (10,000 x *g*) in a microcentrifuge for 2 minutes to remove any residual isopropanol.

7. Transfer the Minicolumn to a new microcentrifuge tube. Apply 50µl (see Table 1) of prewarmed (65–70°C) water or TE buffer (10mM Tris- HCl [pH 7.6], 1mM EDTA) to the Minicolumn and wait 1 minute. (The DNA will remain intact on the Minicolumn for up to 30 minutes.) Centrifuge the Minicolumn for 20 seconds at maximum speed (10,000 x *g*) to elute the bound DNA.
Elute DNA >20kb with water or TE buffer prewarmed to 80°C.

8. Remove and discard the Minicolumn. The purified DNA may be stored in

the microcentrifuge tube at 4°C or –20°C.

9. PROMEGA WIZARD GENOMIC DNA PURIFICATION KIT, PROTOCOL FOR 'ISOLATING GENOMIC DNA FROM PLANT TISSUE

Materials to Be Supplied by the User

- 1.5ml microcentrifuge tubes
- microcentrifuge tube pestle or mortar and pestle
- water bath, 65°C
- water bath, 37°C
- isopropanol, room temperature
- 70% ethanol, room temperature

1. Leaf tissue can be processed by freezing with liquid nitrogen and grinding into a fine powder using a microcentrifuge tube pestle or a mortar and pestle. Add 40mg of this leaf powder to a 1.5ml microcentrifuge tube.

2. Add 600µl of Nuclei Lysis Solution, and vortex 1–3 seconds to wet the tissue.

3. Incubate at 65°C for 15 minutes.

4. Add 3µl of RNase Solution to the cell lysate, and mix the sample by inverting the tube 2–5 times. Incubate the mixture at 37°C for 15 minutes. Allow the sample to cool to room temperature for 5 minutes before proceeding.

5. Add 200µl of Protein Precipitation Solution, and vortex vigorously at high speed for 20 seconds.

6. Centrifuge for 3 minutes at 13,000–16,000 × *g*. The precipitated proteins will form a tight pellet.

7. Carefully remove the supernatant containing the DNA (leaving the protein pellet behind) and transfer it to a clean 1.5ml microcentrifuge tube containing 600µl of room temperature isopropanol.

Note: Some supernatant may remain in the original tube containing the protein pellet. Leave this residual liquid in the tube to avoid contaminating the DNA solution with the precipitated protein.

8. Gently mix the solution by inversion until thread-like strands of DNA form a visible mass.

9. Centrifuge at 13,000–16,000 × *g* for 1 minute at room temperature.

10. Carefully decant the supernatant. Add 600µl of room temperature 70% ethanol and gently invert the tube several times to wash the DNA. Centrifuge at 13,000–16,000 × *g* for 1 minute at room temperature.

11. Carefully aspirate the ethanol using either a drawn Pasteur pipette or a sequencing pipette tip. The DNA pellet is very loose at this point and care must be used to avoid aspirating the pellet into the pipette.

12. Invert the tube onto clean absorbent paper and air-dry the pellet for 15 minutes.

13. Add 100µl of DNA Rehydration Solution and rehydrate the DNA by incubating at 65°C for 1 hour. Periodically mix the solution by gently tapping the tube. Alternatively, rehydrate the DNA by incubating the solution overnight at room temperature or at 4°C.

14. Store the DNA at 2–8°C.

10. PCR PROTOCOL BIOSYSTEMATICS GROUP

When attempting PCR on new DNA or for a new marker for the first time prepare a scaled down (15 µl) reaction to test whether the PCR will actually work. If the reaction works well then scale up your reaction to 50µl.

1. **Thaw** 10x buffer, dNTPs Primer, $MgCl_2$ and BSA (the latter is an optional component of PCR mixes that often improves PCR reactions when used at a concentration of 4mg/ml – stock solution is 10mg/ml – if you use BSA make sure you alter the volume of water so that the final volume of each reaction remains the same). Leave the polymerase at-20^{o}C until you need it. When you have added all other components to the PCR mix take the polymerase out of the freezer and keep it on ice. Use a **fresh stock of MQ water.** The polymerase is stored in glycerol so it is not freezing at -20^{o}C and therefore it is not necessary to thaw the solution. To prevent contamination with other DNA it is important to use fresh autoclaved MQ water for the PCR.

2. **Calculate** the amount of **PCR mixture** needed and record it in your lab book. Below is indicated how much of the different components are used for a typical 50µl PCR reaction. **Multiply** with the number of samples **plus one** sample for a negative control (containing only water and no template DNA), one sample as a positive control and **one** sample to cover loss of PCR mixture due to pipetting errors. For example: when you want to PCR 20 samples, make a PCR mixture for 22 samples.

Depending on the quantity of your **template DNA make dilutions** in MQ water. If you have a concentrated DNA solution dilute it **1:100,** otherwise dilute it **1:10**. To make a 1:10 dilution, add 1 µ**l** l on 9 µ**l** sterile water. It may be easier to pipette 2 µ**l** to 18 µ**l** sterile water. First add water to a new tube than add the DNA because if you have to pipette a small volume it is easier to add it directly into a solution that is already in the tube.

4. Put **2** µ**l** of every **DNA sample** in a 0.2ml **PCR tube** or use a 96 well-multititre plate. Keep DNA on ice. Use 2 µ**l** DNA, 2 µ**l** of a 1:10 dilution or 2 µ**l** of a 1:100 dilution depending on the quantity of your DNA (see above). Always include a **negative control** where you use **2** µ**l of sterile water** instead of DNA. If you get an amplification product in the negative control this will tell you that one or more of your PCR reagents are contaminated with DNA. If you have contamination in your negative control then you will need to re-run the PCR using fresh reagents. Also include a **positive control** where you include DNA from a sample that has worked well in previous reactions

5. **Prepare** the **PCR mixture** in a 1.5ml (2ml) tube **on ice**: Add **reaction buffer**, **$MgCl_2$, dNTPs**, **primer**, **water** and **Taq DNA polymerase** in this order. Mark on a **check list** after adding each component. Leave the polymerase at –20^{o}C until you are ready to add it to the PCR mixture, take it out of the freezer just before adding it to the PCR mixture, keep it on ice and put it back immediately when you are ready.

6. **Mix** the PCR mixture carefully by pipetting carefully up and down. Due to the glycerol the polymerase sinks to the bottom of the tube. It is therefore very important to mix the entire PCR mixture carefully before aliquotting.

7. If necessary, **spin down** briefly to collect the liquid at the bottom of the tube.

Add 48 µ**l of the PCR mixture** to the DNA samples that are already in the PCR tubes. Add the PCR **mix** to one sample and use the same pipette tip to mix it with the DNA. Then use a clean pipette tip to add the PCR mix to the next sample.

9. **Verify your work** after adding the PCR mixture **by comparing the liquid level in the tubes** (they should all be equal). NB: during preparation of PCR reactions it is easy to skip a tube or to add the mixture twice!

10. **Close tubes carefully** with a lid to prevent evaporation during the PCR reaction. If you use a 96-wells multititerplate put a rubber mat to seal the plate. Make sure to use the mat in the right orientation: the rounds have to face down and `points` face up.

11. Place the tubes in the **PCR machine**, and start the following programs depending on which gene you are amplifying:

Check PCR products on gel:

12. Load 5 µl of PCR mix plus 2µl loading dye on **1% agarose gel** to check specificity and yield of the PCR (refer to section 7 for the Gel electrophoresis protocol and section 8 for capturing images using the gel doc system).

Storage of the PCR products:

13. Store PCR samples **well labeled** at -**20OC** afterwards. You can store the PCR fragments at 4OC for a couple of days but make sure the lids are tightly closed! Otherwise you will lose your PCR due to evaporation. Ideally you should clean your reactions immediately after the PCR program has finished (see page 35 for PCR purification).

11. GeneJET PCR Purification Kit

1 Add a 1:1 volume of Binding Buffer to completed PCR mixture (e.g. for every 100 µL of reaction mixture, add 100 µL of Binding Buffer). Mix thoroughly. Check the color of the solution. A yellow color indicates an optimal pH for DNA binding. If the color of the solution is orange or violet, add 10 µL of 3 M sodium acetate, pH 5.2 solution and mix. The color of the mix will become yellow.

2 for DNA ≤500 bp

Optional: if the DNA fragment is ≤500 bp, add a 1:2 volume of 100% isopropanol (e.g., 100 µL of isopropanol should be added to 100 µL of PCR mixture combined with 100 µL of Binding Buffer). Mix thoroughly. Note. If PCR mixture contains primer-dimers, purification without isopropanol is recommended. However, the yield of the target DNA fragment will be lower.

3 Transfer up to 800 µL of the solution from step 1 (or optional step 2) to the GeneJET purification column. Centrifuge for 30-60 s. Discard the flow-through. Note. If the total volume exceeds 800 µL, the solution can be added to the column in stages. After the addition of 800 µL of solution, centrifuge the column for 30-60 s and discard flowthrough. Repeat until the entire solution has been added to the column membrane.

4 Add 700 µL of Wash Buffer (diluted with the ethanol as described on p. 3) to the GeneJET purification column. Centrifuge for 30-60 s. Discard the flow-through and place the purification column back into the collection tube.

5 Centrifuge the empty GeneJET purification column for an additional 1 min to completely remove any residual wash buffer. Note. This step is essential as the presence of residual ethanol in the DNA sample may inhibit subsequent reactions.

6 Transfer the GeneJET purification column to a clean 1.5 mL microcentrifuge tube (not included). Add 50 µL of Elution Buffer to the center of the GeneJET purification column membrane and centrifuge for 1 min.

Note For low DNA amounts the elution volumes can be reduced to increase DNA concentration. An elution volume between 20-50 µL does not significantly reduce the DNA yield. However, elution volumes less than 10 µL are not recommended.

If DNA fragment is >10 kb, prewarm Elution Buffer to 65°C before applying to column.

If the elution volume is 10 µL and DNA amount is ≥5 µg, incubate column for 1 min at room temperature before centrifugation.

7 Discard the GeneJET purification column and store the purified DNA at -20°C.

12. GENBANK ACCESSION

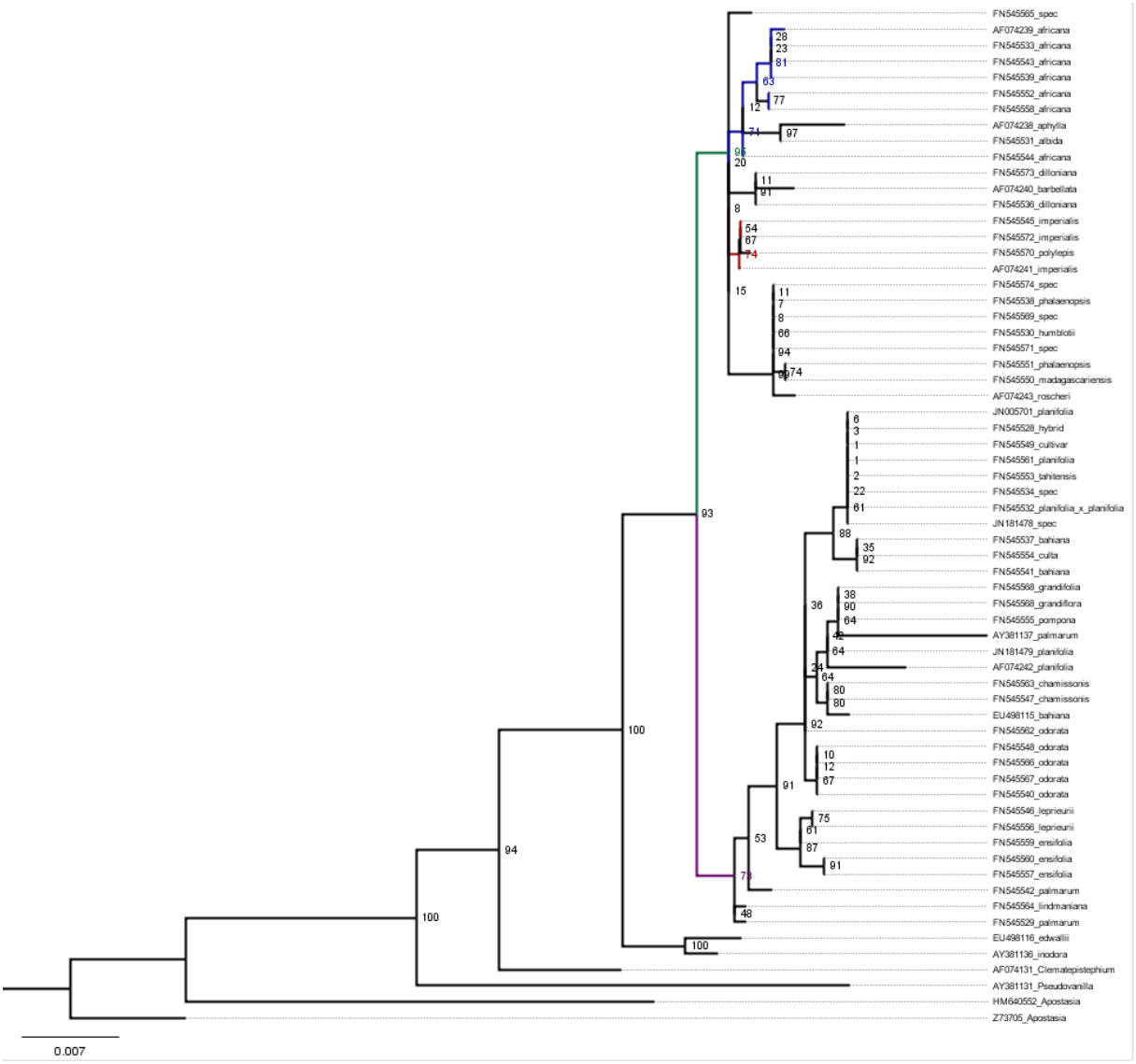
Number	Species	Author	Voucher
JN005701	V. planifolia		SBB-0324
JN181479	V. planifolia		
JN181478	V. spec	Guo, Y.Y., Luo, Y.B., Liu, Z.J., Wang, X.Q.	
AF074243	V. roscheri	Cameron, K.M., Chase, M.W., Whitten, W.M., Kores, P.J., Jarrell, D.C., Albert, V.A., Yukawa, T., Hills, H.G., Goldman, D.H.	
FN545572	V. cf imperialis	Bouetard, A., Lefeuvre, P., Gigant, R., Bory, S., Pignal, M., Besse, P., Grisoni, M.	clone CR0705
AF074241	V. imperialis	Cameron, K.M., Chase, M.W., Whitten, W.M., Kores, P.J., Jarrell, D.C., Albert, V.A., Yukawa, T., Hills, H.G., Goldman, D.H.	
FN545543	V. cf africana	Bouetard, A., Lefeuvre, P., Gigant, R., Bory, S., Pignal, M., Besse, P., Grisoni, M.	clone CR0102
FN545539	V. cf africana	Bouetard, A., Lefeuvre, P., Gigant, R., Bory, S., Pignal, M., Besse, P., Grisoni, M.	clone CR0091
FN545533	V. cf africana	Bouetard, A., Lefeuvre, P., Gigant, R., Bory, S., Pignal, M., Besse, P., Grisoni, M.	clone CR0065
FN545558	V. cf africana	Bouetard, A., Lefeuvre, P., Gigant, R., Bory, S., Pignal, M., Besse, P., Grisoni, M.	clone CR0175
FN545552	V. cf africana	Bouetard, A., Lefeuvre, P., Gigant, R., Bory, S., Pignal, M., Besse, P., Grisoni, M.	clone CR0153
AF074240	V. cf barbellata	Cameron, K.M., Chase, M.W., Whitten, W.M., Kores, P.J., Jarrell, D.C., Albert, V.A., Yukawa, T., Hills, H.G., Goldman, D.H.	Chase O-591
AF074239	V. africana	Cameron, K.M., Chase, M.W., Whitten, W.M., Kores, P.J., Jarrell, D.C., Albert, V.A., Yukawa, T., Hills, H.G., Goldman, D.H.	
AF074238	V. aphylla	Cameron, K.M., Chase, M.W., Whitten, W.M., Kores, P.J., Jarrell, D.C., Albert, V.A., Yukawa, T., Hills, H.G., Goldman, D.H.	
AY381137	V. palmarum	Cameron,K.M.	E. Santo s.n."
FN545529	V. palmarum	Bouetard,A.	clone CR0083
AY381136	V. inodora	Cameron,K.M.	C. McCartney s.n."
FN545544	V. africana	Bouetard, A., Lefeuvre, P., Gigant, R., Bory, S., Pignal, M., Besse, P., Grisoni, M.	clone="CR0103"

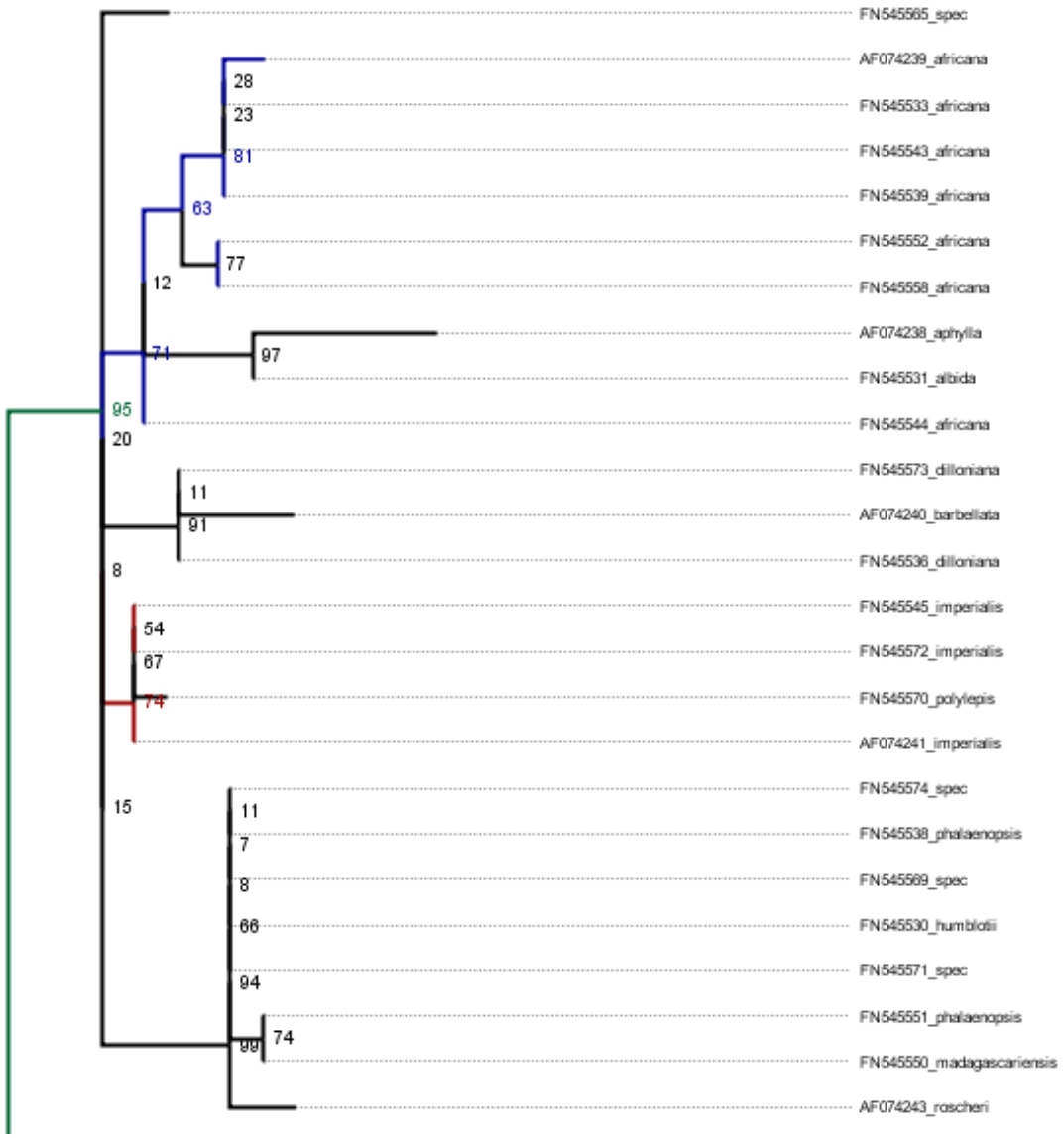
FN545542	V. palmarum	Bouetard, A., Lefeuvre, P., Gigant, R., Bory, S., Pignal, M., Besse, P., Grisoni, M.	clone CR0100
FN545573	V. dilloniana	Bouetard, A., Lefeuvre, P., Gigant, R., Bory, S., Pignal, M., Besse, P., Grisoni, M.	clone CR0794
FN545574	V. spec	Bouetard, A., Lefeuvre, P., Gigant, R., Bory, S., Pignal, M., Besse, P., Grisoni, M.	clone CR0810
FN545571	V. spec	Bouetard, A., Lefeuvre, P., Gigant, R., Bory, S., Pignal, M., Besse, P., Grisoni, M.	clone CR0699
FN545565	V. spec	Bouetard, A., Lefeuvre, P., Gigant, R., Bory, S., Pignal, M., Besse, P., Grisoni, M.	clone CR0067
FN545567	V. odorata	Bouetard, A., Lefeuvre, P., Gigant, R., Bory, S., Pignal, M., Besse, P., Grisoni, M.	clone CR0686
FN545563	V. chamissonis	Bouetard, A., Lefeuvre, P., Gigant, R., Bory, S., Pignal, M., Besse, P., Grisoni, M.	clone CR0666
FN545559	V. cf ensifolia	Bouetard, A., Lefeuvre, P., Gigant, R., Bory, S., Pignal, M., Besse, P., Grisoni, M.	clone CR0177
FN545560	V. ensifolia	Bouetard, A., Lefeuvre, P., Gigant, R., Bory, S., Pignal, M., Besse, P., Grisoni, M.	clone CR0178
FN545555	V. pompona	Bouetard, A., Lefeuvre, P., Gigant, R., Bory, S., Pignal, M., Besse, P., Grisoni, M.	clone CR0169
FN545553	V. tahitensis	Bouetard, A., Lefeuvre, P., Gigant, R., Bory, S., Pignal, M., Besse, P., Grisoni, M.	clone CR0164
FN545551	V. phalaenopsis	Bouetard, A., Lefeuvre, P., Gigant, R., Bory, S., Pignal, M., Besse, P., Grisoni, M.	clone CR0146
FN545549	V. cultivar	Bouetard, A., Lefeuvre, P., Gigant, R., Bory, S., Pignal, M., Besse, P., Grisoni, M.	clone CR0131
FN545547	V. chamissonis	Bouetard, A., Lefeuvre, P., Gigant, R., Bory, S., Pignal, M., Besse, P., Grisoni, M.	clone CR0115
FN545568	V. cf grandiflora	Bouetard, A., Lefeuvre, P., Gigant, R., Bory, S., Pignal, M., Besse, P., Grisoni, M.	clone CR0693

13. PHYLOGENIES OF RBCL REGION FOR VARIOUS COMBINATIONS OF SEQUENCES

Colours for all figures: purple: American clade; green: African clade: red: imperialis clade; blue: africana clade.

PHYLOGENETIC TREE OF BASIS ALIGNMENT

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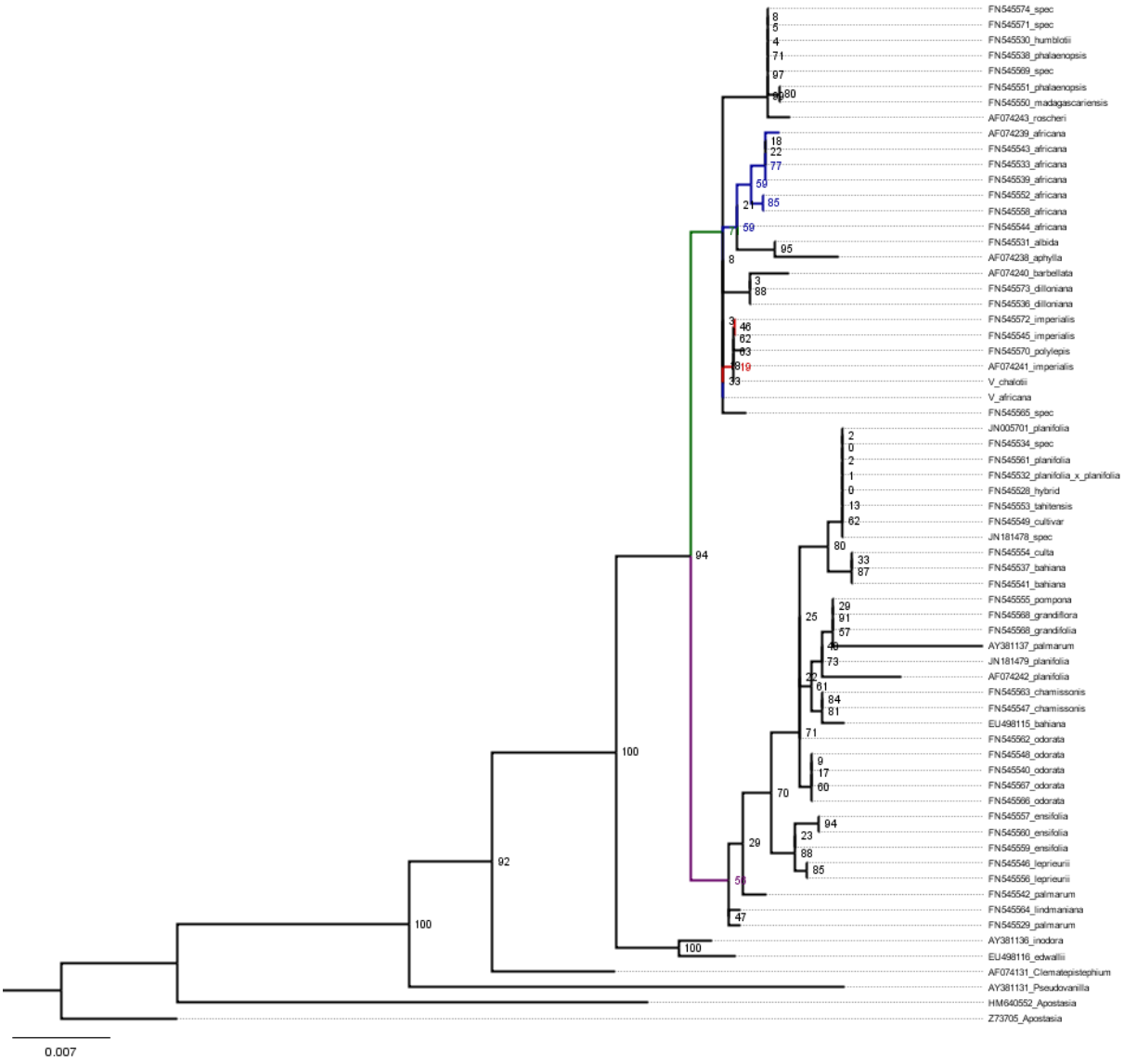
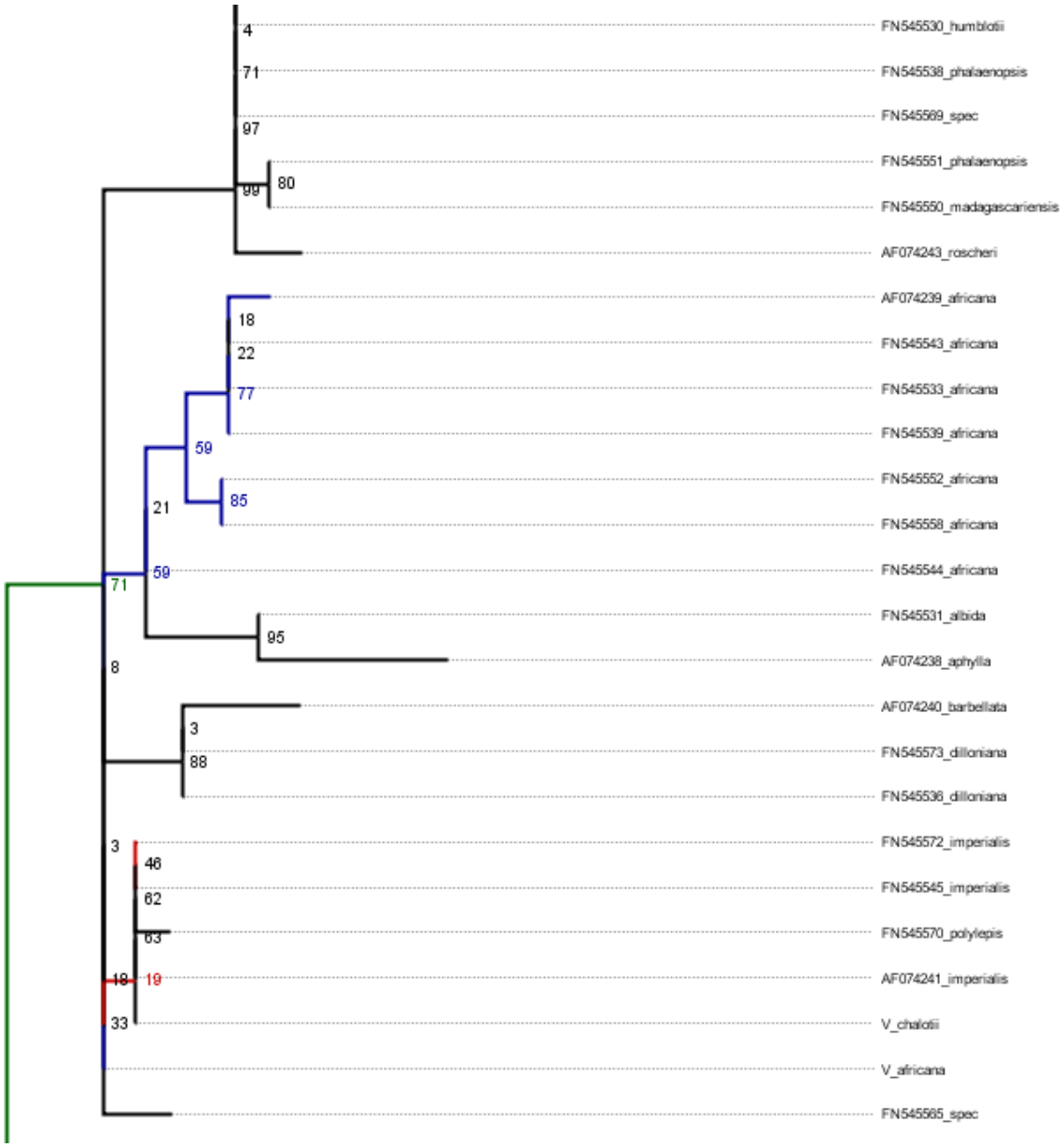
PHYLOGENETIC TREE OF BASIS ALIGNMENT PLUS DNA SEQUENCES OBTAINED WITH HISTORICAL DNA METHODS

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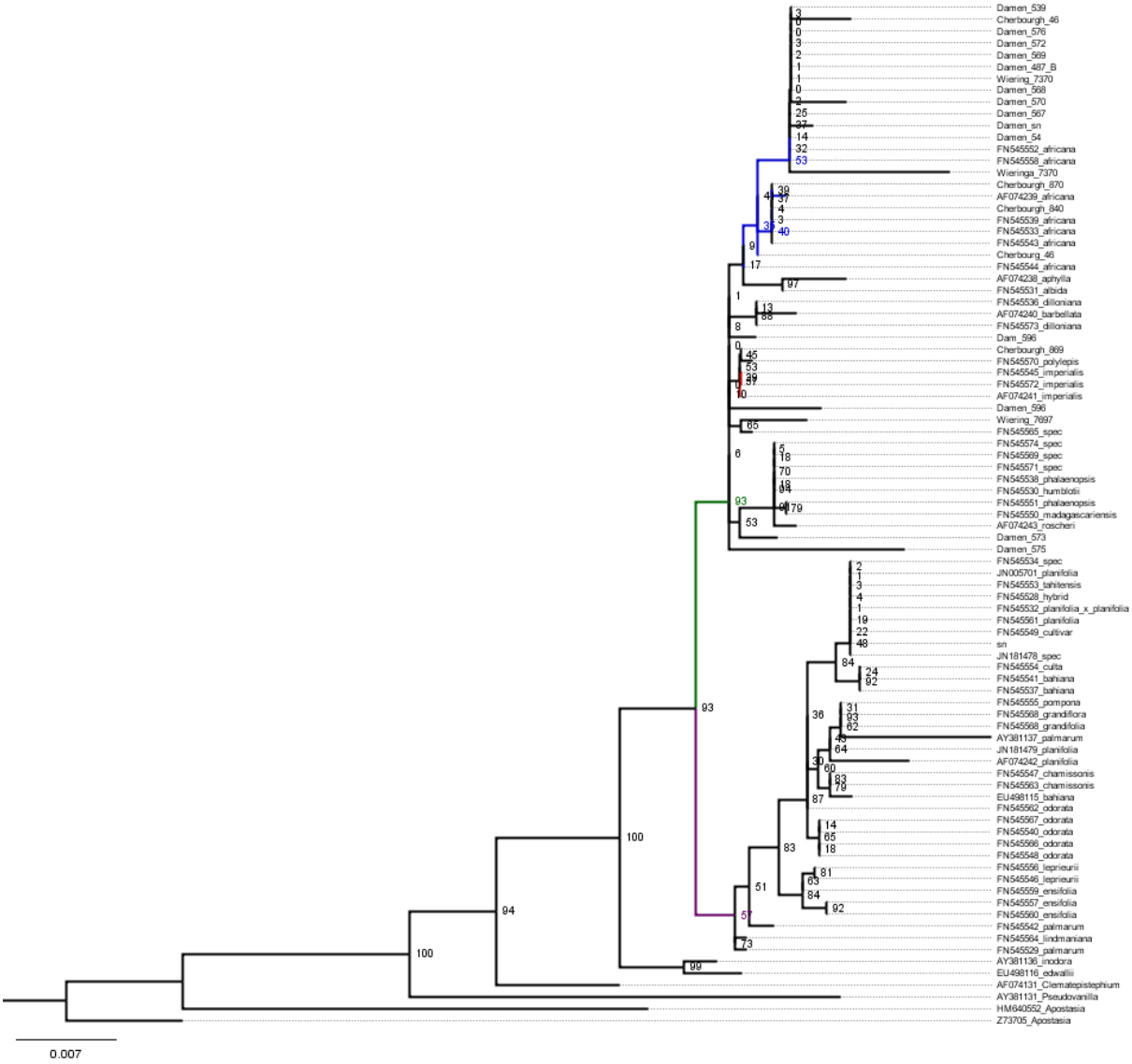
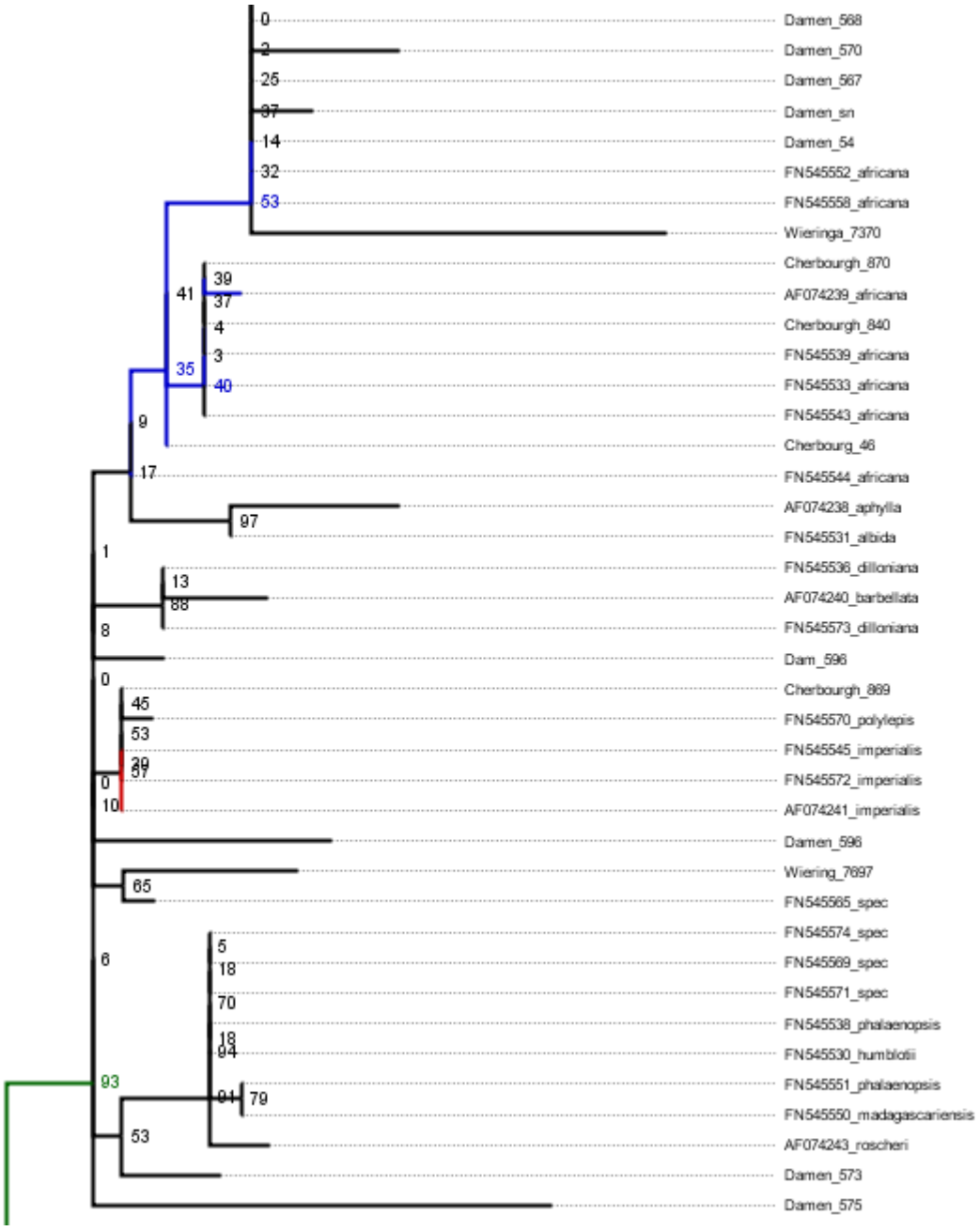
PHYLOGENETIC TREE OF BASIS ALIGNMENT PLUS SEQUENCES OBTAINED WITH REGULAR EXTRACTION METHODS (BOTH PRIMERS)

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