Systematics, epidermal defense and bioprospecting of wild orchids

Richa Kusuma Wati

SYSTEMATICS, EPIDERMAL DEFENSE AND BIOPROSPECTING OF WILD ORCHIDS

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This thesis is dedicated to my sons, Rayyan and Aidan

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Introduction

1.1 Indonesia and its biodiversity

Indonesia is a tropical archipelago between Asia and Australia, and the Pacific and Indian Oceans with more than 17,000 islands. Geologically, Indonesia, is situated at the boundaries of three major plates: Eurasia, India-Australia, and the Pacific-Philippine Sea. Indonesia is bordered by tectonically active zones and included in the Circum-Pacific Belt that is also called the Ring of Fire (Hall, 2009). Based on Maryanto and Higashi (2011), Indonesia is classified into seven bio-regions: Sumatra, Java and Bali, Kalimantan, Sulawesi, the Lesser Sunda Islands, Moluccas, and Papua. Indonesia is the second-highest biodiversity hotspot after Brazil for terrestrial flora and fauna and even the highest when combined with marine biodiversity (Widjaja et al., 2014).

Indonesia is known to harbor 25% of all flowering plant species worldwide, of which 40% consists of Indonesian endemics (Ministry of Environment and Forestry of Indonesia, 2015). For orchids, more than 5000 species out of the 25,000 species in the world occur in Indonesia (Banks, 2004). Recent surveys record a total of 7,622 orchid species distributed among the seven bio-regions of Indonesia (see Table 1.1). According to this survey, the highest orchid diversity is found in Kalimantan, but O'Byrne (1994) reported New Guinea is estimated to have more than 3,500 species of orchids and the Lesser Sunda Islands are underexplored as well.

A very popular group of orchids in Indonesia, belonging to subfamily Epidendroideae and subtribe Coelogyninae, are the Necklace orchids. These are common epiphytes found in tropical Asia and the Pacific, occurring from Sri Lanka, through India, Southeast Asia, the Malay Archipelago, Taiwan, Japan, and the tropical Pacific islands east to Samoa (Pridgeon et al., 2005). The term Necklace orchids is based on a characteristic of the most popular species: a long, pendant, multi-flowered inflorescence that resembles a necklace (Figure 1.1). Most species have small (<1 cm in diameter) to medium-sized (<5 cm in diameter)

flowers with a sweet scent. The subtribe comprises 21 genera and 755 species in well-studied genera such as *Coelogyne* and *Dendrochilum* and lesser-known genera such as *Glomera*. Many species in this subtribe are used in traditional medicinal practices in China and Himalaya, especially from the genera *Bletilla, Coelogyne, Dendrochilum, Otochilus, Pholidota, Pleione* and *Thunia* (Singh and Duggal, 2009; Subedi et al., 2011; Pant and Raskoti, 2013; Teoh, 2016).

Table 1.1. Number of species distributed among seven bio-regions of Indonesia (Widjaja et al., 2014)

Bioregion	Number of species
Java	1604
Kalimantan	2,769
Lesser Sunda Islands	197
Moluccas	755
Papua	2,715
Sulawesi	1,083
Sumatra	2,001

1.2 Bogor Botanic Gardens and their role in species discovery

Bogor Botanic Gardens, formerly known as ‘s Lands Plantentuin, was established in 1817 in the Dutch colonial era by **Caspar Georg Carl Reinwardt**. The garden’s main purpose at that time was to become a productive repository and testing ground for the acclimatization of plants from the Indonesian archipelago and to serve as an important entry point for commercially exploitable plants from Europe and other colonial gardens in Asia and South America (Weber, 2014). The gardens are situated behind the governmental palace and next to the Ciliwung river supplying the plants in the gardens with sufficient water. Reinwardt stressed the economic benefit of the immense fertility and extreme diversity of Indonesia’s biodiversity and the necessity to carry out a meticulous examination of plants, animals, and minerals in their original site to identify and exploit these in a productive way (Reinwardt, 1823).

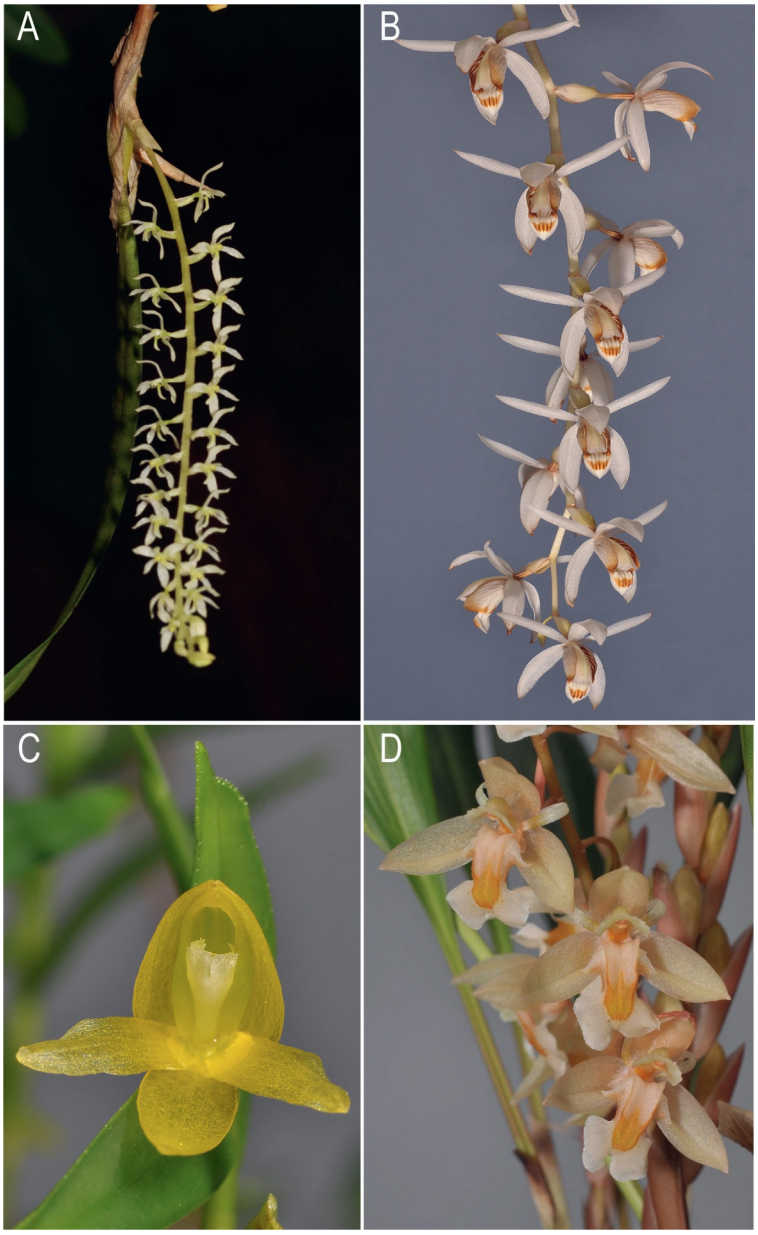

Figure 1.1. Some examples of genera in the Necklace orchids (Coelogyninae) with typically long and pendant inflorescences. A. *Dendrochilum pallidiflavens* Blume, B. *Chelonistele sulphurea* (Blume) Pfitzer, C. *Aglossorhyncha lucida* Schltr., and D. *Coelogyne swaniana* Rolfe. Photograph A by Hendrik A. Pedersen. Photographs B-D by Rogier van Vugt.

Extensive travels and expeditions were undertaken to enrich the botanical gardens with new plant species. **Carl Ludwig Blume** organized expeditions in West and Central Java in 1822 for this purpose. Other expeditions to different islands were carried out by **Alexander Zippelius** in 1828, **Pieter Willem Korthals** in 1823, **Eltio Alegondas Forsten** in 1840, **Heinrich Zollinger** in 1842-1847, **Johannes Elias Teijsmann** in 1853-1877, **Johannes Jacobus Smith** in 1891-1900, **Willem Marius Docters van Leeuwen** in 1913-1929, **Cornelis Gijsbert Gerrit Jan van Steenis** in 1927-1929 and **Reinier Cornelis Bakhuizen van Den Brink** in 1917-1935. In the first catalog of the gardens, published by Blume in 1823, a total of 912 plant species were listed. When Teijsmann was appointed as curator to manage the Bogor Palace and Bogor Botanic Gardens in the period 1830-1868, the plant collection rapidly expanded. **Justus Karl Hasskarl** was appointed to assist Teijsmann with re-arranging the collection according to taxonomic order. These changes made the Bogor Botanic Gardens scientifically more valuable and attractive as a research station for international botanists to conduct research on tropical plants. Consequently, the second catalog from 1844 recorded a total of 150 species of ferns, 25 species of gymnosperms, 510 species of monocots, and 2,200 species of dicots.

Four palm oil seeds (*Elaeis guineensis* Jacq.), brought from Africa, were planted in the gardens in 1848. These oil palm individuals became the ancestors of the oil palms now widely spread throughout plantations in Indonesia and other countries in Southeast Asia (Price et al., 2007). Quinine (*Cinchona calisaya* Wedd.), imported from Bolivia, was planted in the Cibodas Botanic Gardens (*Bergtuin te Cibodas*) in 1852 upon their establishment (Teijsmann, 1861). Teijsmann also undertook many excursions to Japan, China, India, Sri Lanka, Brazil, and Australia to further expand the plant collection. After Teijsmann, **Rudolph Scheffer** was appointed as director of the Bogor Botanic Gardens. He founded the first scientific journal of the gardens called *Annales du Jardin Botanique de Buitenzorg*, in which many new species of tropical plants were described, including orchids. Scheffer was succeeded by **Melchior Treub** in 1880. Under his directorship, taxonomic research at the gardens was expanded with physiology and genetics. Treub also improved the aesthetic value of the gardens by creating beautiful thematic gardens to attract non-scientific visitors (Sukarya and Witono, 2017).

Figure 1.2. The difference between past and current education on biodiversity in Indonesia. A. Class room of **Leendert van der Pijl** in Bandung, where he worked from 1927 until 1947 as biology teacher, in which the jungle was brought to the pupils. B. Excursion let by **Yoga Dwipayana** in the Bogor Botanic Gardens in 2015, where the pupils are brought to the jungle. Photographs kindly provided by Priska Becking and the Bogor Botanic Gardens Flora Tourism Team.

From species discovery to conservation

After the independence of Indonesia in 1956, the Bogor Botanical Gardens, now called Pusat Konservasi Tumbuhan Kebun Raya, were led by **Sudjana Kasan**. Three branches were gradually established: next to the Cibodas Botanical Gardens in West Java (1862), the Purwodadi Botanical Gardens (1941) in East Java, and Eka Karya Botanical Gardens in Bali (1959) were founded. The four Botanic Gardens under the Indonesian Institute of Sciences were called Indonesian Botanic Gardens (IBG). The aim of the gardens gradually evolved from plant collection into plant conservation, research, education (Figure 1.2), ecotourism, and environmental services, the latter especially to curb deforestation (Indonesian Presidential Decree no. 93 on Botanic Gardens, 2011). In this period, **Leendert van der Pijl** and his students discovered many new pollinators of Indonesian plants, especially orchids (van der Pijl and Dodson, 1966). Under the directorship of **Irawati**, the first female director, the orchid research, especially in vitro culture of rare Indonesian orchids, was further developed (Figure 1.3). Since the 19th century, the Bogor Botanic Gardens also became known as a very famous tourist destination. In 2019 the gardens were visited by a total of 1,135,495 local visitors and 15,749 foreign visitors. The gardens have become a national legacy, with an important role in the development and progress of botanical, agricultural, and plantation sciences in Indonesia. Several new research institutions were also initiated by the Bogor Botanic Gardens such as the Bibliotheca Bogoriensis, Museum Zoologicum Bogoriense, Ocean Research Institution, Bogoriense Herbarium, Nature Preservation Institution, Flora Malesiana Foundation, and Microbiological Institution and Academy of Biology. The current generation of researchers of the Bogor Botanic Gardens produced seeds from hand pollination of *Amorphophallus titanum* (Becc.) Becc. (Sudarmono et al., 2016) and successfully developed a protocol for initiating ex situ flowering of *Rafflesia patma* Blume (Mursidawati et al., 2015).

From conservation to education and environmental services

On the international scale, IBG agreed to implement the Global Strategy for Plant Conservation (GSPC) with a total of 16 targets to save plants (Davis, 2008).

According to target 8 of the GSPC, between 2011 and 2020, a minimum of 75% of all endangered plant species should be collected in their original country, and 20% of them should be reintroduced. In the four separate botanic gardens, IBG was only curating a total of 21.5% of all Indonesian threatened plants in four botanic gardens (Purnomo et al., 2015). To create more space and care for all collected plants, Bogor Botanic Gardens, therefore, initiated the establishment of new botanical gardens under a regional government, called the Regional Botanic Gardens (RBG). The target is to establish a total of 47 Botanic Gardens based on the Terrestrial Ecoregion and WWF Ecoregion classification (Olson et al., 2001). In 2012, IBG and RBG managed a total of 24% of Indonesian threatened plants (Purnomo, et al., 2015). So far, a total of 44 additional Botanic Gardens have been established in 23 provinces, 5 Botanic Gardens under LIPI, 37 Botanic Gardens under Regional Governments, and 2 Botanic Gardens under the University of Haluoleo and Institute of Technology Sumatra (ITERA). This initiative will help to conserve the endangered native plants of Indonesia.

1.3 Orchid research in the Bogor Botanic Gardens

Probably the very first study of orchids in the Bogor Botanic Gardens was carried out by Blume, who described a large number of new orchid species from West and Central Java. Gaining advantage from his appointment as inspector of smallpox vaccinations, Blume could observe the flora of many parts of Java. One of the orchid genera published by him was *Glomera*, which was described in 1825 in *Blume's Bijdragen tot de flora van Nederlandsch Indië* with *G. erythrosma* Blume as type, the only species occurring in Java. *Glossorhyncha*, a close relative, now included in *Glomera*, was described in 1891 by **John Ridley** in The Journal of the Linnean Society Botany with *G. amboinensis* as type, the first described species from the Moluccas (Wati, van Vugt and Gravendeel, 2018). Blume's most important contribution to orchidology was constructing a system of affinity for tropical orchids. In his *Catalogus van eenige der merkwaardigste zoo in- als uitheemsche gewassen te vinden in's lands plantentuin te Buitenzorg*, published in 1823, the orchid collection of the Bogor Botanic Gardens at that time was listed. It included species used in traditional medicinal practices such as *Acriopsis javanica* Reinw. ex Blume, of which extracts of the pseudobulbs are used to treat

earache and fever by the Sundanese people. Other economically important species included *Cypripedium javanicum* Reinw. ex Lindl., a synonym of *Paphiopedilum javanicum* (Reinw. ex Lindl.) Pfitzer, an Indonesian endemic, nowadays used as the parent of many artificially created orchid hybrids. Other discoveries contributing to commercial exploitation of orchids were made by Teijsmann, who introduced artificial pollination of *Vanilla planifolia* Andrews plants in Java for the production of vanilla pods. Smith and **Rudolf Schlechter** described many new orchid species collected throughout Indonesia.

Around 1900, the Foreigner's Laboratory, later called Treub Laboratorium, was built inside the Bogor Botanic Gardens to attract researchers from all over the world. Treub studied orchid embryology, seeds, and seedlings there and proposed the term protocorm (Treub, 1890) for early-stage orchid seedlings (Yam and Arditti, 2009). He also performed the first histochemical study of the orchid embryo (Treub, 1879). In this same laboratory, **Hans Fittings** studied pollinia of *Phalaenopsis* in 1909 and saw that pollen tubes release a substance that induces post pollination phenomena such as ovule development (Fitting, 1909a, 1909b, 1910). This substance was later discovered to be auxin by **Frits Warmolt Went** (Went, 1926), who worked in the laboratory from 1927 to 1933. **Docters van Leeuwen** studied the seed dispersal of *Epipogium roseum* (D.Don) Lindl., a saprophytic orchid there (Docters van Leeuwen, 1937). He also observed pollination of *Dendrobium hasseltii* (Blume) Lindl. by birds on a mountain summit in Java, autogamy in the genus *Myrmerchis* (van der Pijl and Dodson, 1966) and ant dispersal of orchid seeds with elaiosomes (van Leeuwen, 1929, Wati et al. in prep.). The intimate relationship between orchids and fungi was first studied by **Hans Burgeff** during his visit to the laboratory from 1927 to 1928. Between 1939 and 1955, **Jacoba Ruinen**, the first female orchidologist at the Bogor Botanic Gardens, coined the term epiphytosis for the harmful effects of root-associated fungi of epiphytic orchids to the branches of their host trees, that were revealed by a series of elegant experiments carried out in the laboratory and gardens (Ruinen, 1953).

During 1990-2000 many orchid researchers, amongst others from the Royal Botanic Gardens, Kew, Hortus botanicus Leiden, and Singapore Botanic Gardens visited the gardens to study the plant collections. **Jeffrey James Wood** and **Phillip James Cribb** published their Orchids of Borneo series (1997-2004), and

James Boughtwood Comber his Orchids of Java (1990), and Orchids of Sumatra (2001) books. **Eduard de Vogel** and **André Schuiteman**, next to publishing many scientific articles, produced an innovative CD-ROM series (http://www.orchidsnewguinea.com/). **Peter O'Byrne** published his 'A to Z of South East Asian Orchid Species' guide for orchid novices, and **Barbara Gravendeel** wrote her PhD thesis on *Coelogyne* and allies.

In the 21st century, a tissue culture laboratory was built next to the orchid greenhouse to maintain the collection, supply it with seedlings, and exploit it commercially. Currently, **Djauhar Asikin**, **Dwi Murti Puspitaningtyas**, **Sofi Mursidawati**, **Elizabeth Handini**, **Yupi Isnaini**, **Eka Martha Della Rahayu**, **Vitri Garvita Gandadikusumah**, and myself manage and study this collection. Important discoveries include successful propagation of endangered orchids such as *Phalaenopsis gigantea* J.J.Sm., *P. violacea* H. Witte, *P. celebensis* H.R. Sweet, *P. javanica* J.J.Sm., *Paraphalenopsis serpentilingua* (J.J.Sm.) A.D. Hawkes, and *P. laycockii* (M.R. Hend.) A.D. Hawkes. The regeneration of protocorm and acclimatization of *Cymbidium hartinahianum* J.B. Comber & Nasution, an orchid species that was assumed to be extinct in the wild after its small native area was converted into a potato field, was successfully developed (Handini et al., 2018). Bioprospecting of medicinally used Indonesian orchids was applied to find new sources of bioactive compounds (Wati et al., in press). Collaboration with fellow researchers such as **Aninda Retno Utami Wibowo** from the Eka Karya Botanical Gardens and **Dewi Pramanik** from the Indonesian Agency for Agricultural Research and Development provides new insights in the evolutionary origin of highly specialized orchid floral structures such as the mentum (Pramanik et al., 2020).

1.4 Orchid illustrations: from elite art to commercial products and social media for everyone

Smith and Schlechter illustrated their taxonomic descriptions themselves by making black and white pencil drawings while collecting plants in the field. In the 18th century, these were the only resources available, and their drawings are of high scientific value. They lack important diagnostic characteristics, though, such as plant habit and flower color. Fortunately, Smith and Schlechter were

sometimes assisted by professional botanical illustrators, such as **Natadipoera**, a member of the royal family of the Talagamanggung Kingdom in Majalengka, who illustrated nine species of *Glomera* and many species from other orchid genera. **Mas Kromohardjo** illustrated species in the seven volumes of Smith's *Orchideen von Java*, published between 1905 and 1939. Nowadays, ecotourism hugely contributes to science by displaying high-quality photographs of species in the wild on social media such as Facebook, Instagram, Pinterest, Pbase, and Smugmug, in which important details such as flower and leaf color are disclosed (Figure 1.4). A new generation of botanical artists such as **Esmée Winkel** skillfully combines art with science. **Janneke Brinkman** turned her art into a wide array of commercial products (www.jannekebrinkmanshop.com). **Eunike Nugroho** and **Jenny Kartawinata** founded the Indonesian Society of Botanical Artists (IDSBA) in 2017. Its members are botanical artists, illustrators, botanists, researchers, and hobbyists. This organization aims to ensure the continuation of botanical art in Indonesia and increase awareness of Indonesian biodiversity.

1.5 Current challenges for orchid collection management

Management of Indonesian orchid collections ex situ has its challenges. Most of the orchids are obtained from the wild during explorations throughout the archipelago. After freshly collected orchids arrive in one of the branches of the Indonesian Botanic Gardens, the acclimatization process starts. Acclimatization will determine if plants survive in a new environment that is very different from the native habitat. During this process, many orchids die since it is very hard to imitate the macro- and microclimate of the native habitat in a nursery (Trimanto and Rahadiantoro, 2017). Even when the orchids survive, many of them will never flower, making it very difficult to identify the specimens to species level since traditional identification keys often rely on floral characters. The orchid collection in the Bogor Botanic Gardens currently contains 6,004 specimens, consisting of 499 species from 94 genera, of which 1,223 could not yet be identified to the species level (Wati and Mursidawati, 2015). To speed up identification, DNA barcodes from living collections can be linked to data generated from herbarium specimens (Wati et al., in press). Species identification can update information on geographic distributions and answer other questions on the ecology and conservation biology

of endangered species, needed to assign priority areas (Guisan and Zimmermann, 2000).

Another challenge in any living orchid collection, but especially in humid tropical regions, is the protection of plants against herbivores and diseases. Especially snails and slugs are major pests of living orchid collections, either in situ or ex situ. After decades of unsustainable chemical spraying, the use of chemical pesticides is now increasingly legally banned, and bio-based alternatives are called for (Subedi et al., 2011; Wati et al. in prep.).

1.6 Future directions for scientific exploration of living orchid collections

Plants have been used as the basis of traditional medicine systems as early as 2600 BC (Cragg and Newman, 2013). Many modern drugs have been developed from plant bioactive compounds. Over the past decade, an increase in drug-resistant microbes urged for the finding of new antimicrobial compounds to replace current antimicrobial agents. It is estimated that only 6% of all plant species have been screened for biological activity so far, and only 15% of these have been tested phytochemically (Verpoorte, 1998). New time-efficient and systematic approaches are needed to unlock the potential of plants in health-care (Ernst et al., 2016). Plants with medicinal properties are usually found more frequently in certain families and are not randomly distributed throughout the Angiosperm Tree of Life (Moerman, 1991; Saslis-Lagoudakis et al., 2011). The orchid family is long known for its medicinal properties (Lawler, 1984; Singh and Singh, 2012). Bioprospecting offers an effective approach, combining a phylogeny with ethnobotanical knowledge to find potential new sources of medicines, but the availability of ethnobotanical data is essential. Most of the ethnobotanical data for orchid uses come from Traditional Chinese Medicine (TCM), and Himalayan sources, but very few orchid uses have yet been recorded for Indonesian species. Exploiting indigenous knowledge is a promising new way of co-financing the maintenance of living orchid collections. Elsewhere in Asia, such as in Bhutan, patent rights of a traditional face cream

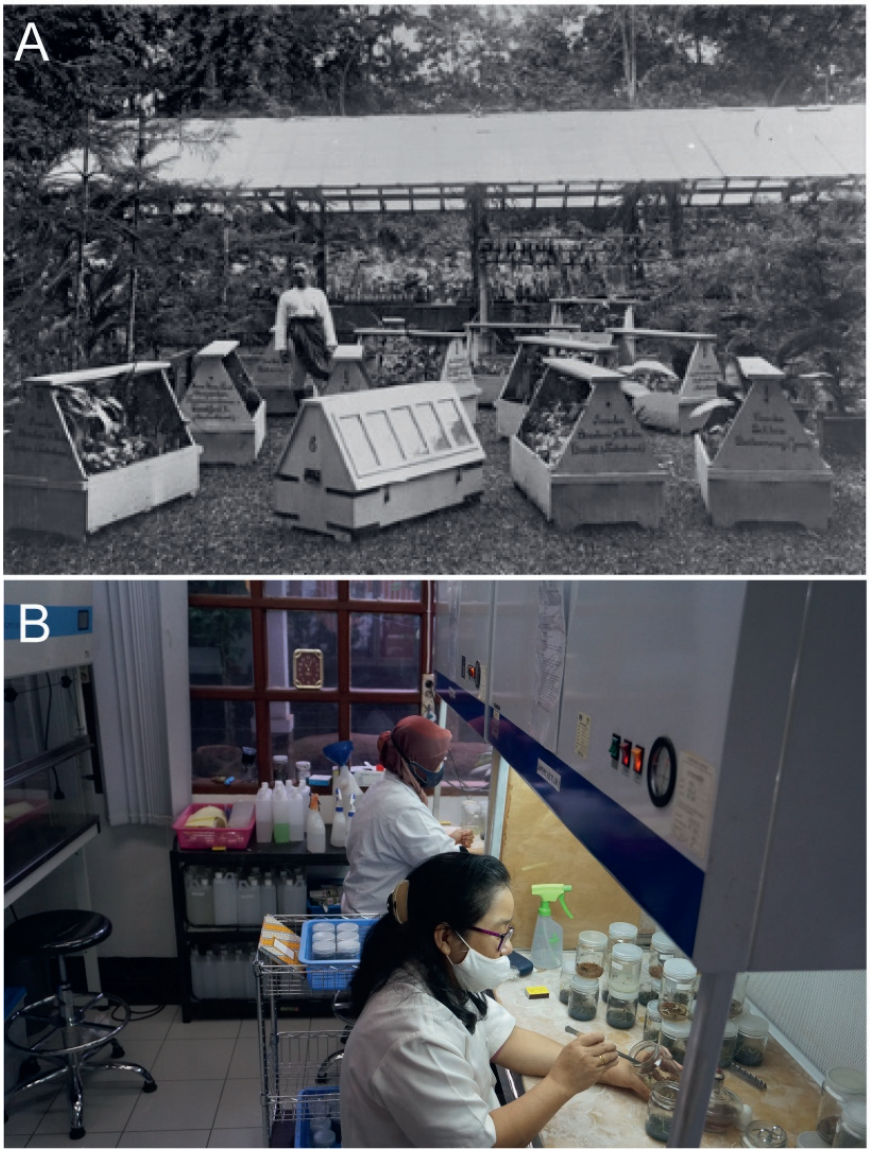

Figure 1.3. The difference between past and current research in the Bogor Botanic Gardens. A. Many new varieties of commercially important plants were developed in the Bogor Botanic Gardens between 1850 and 1890. Cuttings of these new cultivars were sent in large quantities to companies using a *Wardsche box* (Boomgaard and Dijk, 2001). Photograph from the Tropenmuseum Collections. B. Two of the current female researchers producing bottled seedlings of threatened species in the orchid collection. Photograph by Sofi Mursidawati.

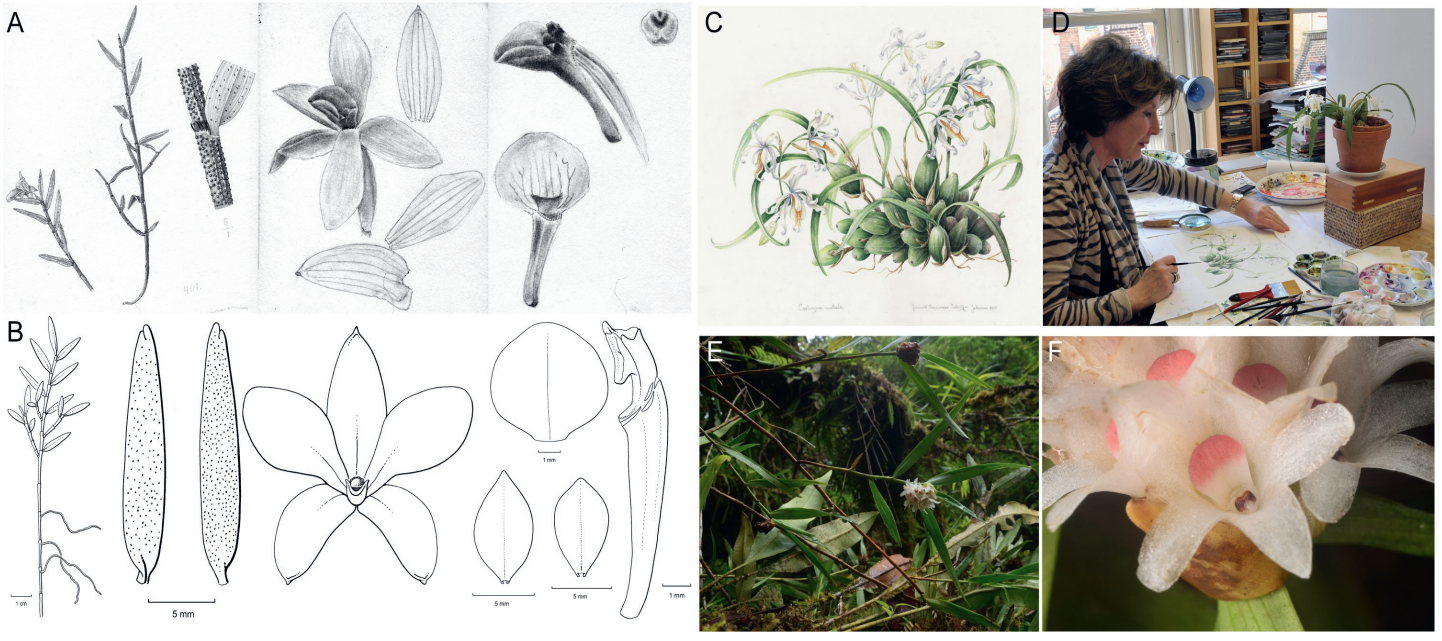

Figure 1.4. An impression of illustrations of Necklace orchids made over the past two centuries. A. Black and white pencil drawing of *Glomera amboinensis* made by Johannes Jacobus Smith to illustrate species descriptions in his publication in the journal *Bulletin du Département de l'Agriculture aux Indes Néerlandaises*. B. Scientific illustration of key diagnostic characters of *G. amboinensis* made by Esmée Winkel for a publication in the journal *Phytokeys*. C. Artistic botanical drawing of *Coelogyne cristata* made by Janneke Brinkman in 2013 for use in commercial products. D. Photograph of Janneke, making the drawing displayed under C, made by Rogier van Vugt. E-F. Photographs of *Glomera montana* in the wild in the Solomon Islands, made by Hsu Tian-Chuan and uploaded in the image hosting platform Flickr.

made from extracts of *Cymbidium erythraeum* Lindl. support economic growth. This ambition was also voiced by the Indonesian Science Fund focus area of Life, Health, and Nutrition to utilize the Biodiversity of Indonesia and develop it into medicine and sources of nutrition.

Aims of the thesis

In this thesis, I targeted the Necklace orchids, with special emphasis on the genus *Glomera*, a highly overlooked and understudied genus with 169 species recorded to date, distributed over Indonesia, Papua New Guinea, and the Philippines. To facilitate an increase in current knowledge on the distribution of all the species of *Glomera*, I made an illustrated, interactive, and bilingual identification key for the entire genus (**Chapter 2**). The online identification key provides easy-access to the detailed information of each species for all users with different knowledge levels. To solve the challenging problem of identification, I developed a DNA barcoding method for the identification of non-flowering specimens in living collections of botanic gardens (**Chapter 3**). Identification is important to update distribution data that could improve the current conservation plan of Indonesian orchids. To improve in situ and ex situ conservation and understanding of orchid defense against herbivory, I investigated the function of the epicuticular properties of orchid leaves by measuring attachment forces of snails (**Chapter 4**). To explore the potential of new plant-based drugs in the light of growing antibiotic resistance worldwide, I carried out bioprospecting of medicinally used Necklace orchids (**Chapter 5**).

Outline of the thesis

The Necklace orchids (Coelogyninae) are an orchid subtribe in subfamily Epidendroideae, comprising 21 genera and over 680 species, that are widely spread throughout Southeast Asia, including Indonesia (Pridgeon et al., 2005). The most overlooked and understudied genus of this subtribe is *Glomera*, which occurs in New Guinea, Papua, the Moluccas, Java, Vanuatu, and the Philippines. The genus is characterized by an elongated, often branching rhizome with many leaves that are enveloped by sheaths at the base. The inflorescences are usually single- or

few-flowered. The flowers are mostly white, but some are orange, salmon-pink, or green. Most species are epiphytes in lowland or montane rainforest up to subalpine environments (Wati, van Vugt and Gravendeel, 2018).

To increase awareness for this genus, we present in Chapter 2 a bilingual interactive identification key built in the Linnaeus platform, available online with detailed species descriptions, distribution maps, illustrations, and color photographs of living specimens. The platform encourages citizen scientists to contribute photographs for updating distribution data.

While studying herbarium material from different species of *Glomera* deposited in the collection of Naturalis Biodiversity Center, we were allowed to extract DNA from type specimens to help identify non-flowering specimens in living orchid collections in various botanic gardens. In Chapter 3, we developed DNA barcoding of type specimens and living collections as a method for rapid identification of non-flowering specimens in botanic gardens. We obtained permission to perform destructive DNA extraction of type specimens from the herbarium of Naturalis Biodiversity Center and Herbarium Bogoriense. We managed to fully sequence the nrITS region of these specimens and match these with DNA barcodes obtained from fresh sterile collections. With this method, several sterile specimens in the living collections could be identified to species. However, several other specimens could not yet be matched to any type specimens, indicating that these likely belong to species new to science. Future explorations in their geographical area of origin might retrieve flowering specimens needed for the formal description of these species.

In addition to the difficulties of identification of living collections, keeping them alive is a challenge as well, mainly due to high levels of herbivory, both in situ as well as ex situ. To better understand orchid defenses against snail herbivores, in Chapter 4, we carried out experiments on attachment forces of snails against the epicuticular leaf properties of four different orchid species to understand how different orchids defend themselves against herbivores. For this purpose, a custom-made centrifuge was designed, consisting of a turntable equipped with a synchronized strobe. Two differently shaped snails were used in the experiments. We found that terrestrial and epiphytic orchids have different epicuticular structures to defend themselves against herbivores.

For commercial exploitation of orchid collections, we carried out a bioprospecting analysis of Indonesian orchids in Chapter 5. Ethnobotanical data from orchids used in China and Himalayan were applied to an expanded phylogeny of the Necklace orchids that included Indonesian species to find new potential sources for bioactive compounds with antimicrobial properties. Two different methods are generally usually used for medicinal properties classification, the Economic Botany Data Collection Standard (EBDCS) and the Biological Response method. We discovered that the EBDCS classification is less effective in discovering the potential of bioactive compounds as compared with the biological response method that detected a wider group of potential medicinal Necklace orchid species. In Chapter 6, I discuss further steps to apply the findings presented in my PhD thesis for conservation and utilization of collections of Indonesian orchids in the Bogor Botanic Gardens and beyond.

Systematics

Chapter 2

A Linnaeus NG interactive key to the species of *Glomera* (Orchidaceae, Coelogyninae) from Southeast Asia

Richa Kusuma Wati, Rogier R. van Vugt, Barbara Gravendeel

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Abstract. We present a multilingual interactive key available online (https://glomera.linnaeus.naturalis.nl) that can be used on any web browser without the need for installing additional software. The key includes 169 species of *Glomera*, a genus within the necklace orchids (Coelogyninae) not yet comprehensively treated in any recent field guide or web-based survey. With this key, plants can be identified using a combination of vegetative and floristic characters in addition to distribution and ecology as a first step to further taxonomic revisions. We urge anyone with an interest in wild orchids in Southeast Asia to contribute new observations to update current information on the distribution of these overlooked plants as a first step for a taxonomic revision and to gain more insight into their conservation status.

Abstrak. Studi ini menyajikan kunci interaktif multibahasa yang dapat diakses secara online (https://glomera.linnaeus.naturalis.nl) dan dapat digunakan pada berbagai jenis peramban web tanpa perlu menggunakan aplikasi tambahan. Kunci pengukur ini terdiri dari 169 jenis *Glomera* dalam genus anggrek kalung (Coelogyninae) yang belum pernah dibahas secara menyeluruh dalam panduan lapangan atau survei berbasis online. Tumbuhan dapat diidentifikasi menggunakan kombinasi karakter vegetatif dan bunga juga distribusi dan ekologi sebagai langkah pertama untuk revisi taksonomi lebih lanjut. Kami menghimbau bagi orang yang mempunyai ketertarikan dengan anggrek pembohong di Asia Tenggara untuk berkontribusi memberikan informasi terbaru dan menambahkan distribusi data dari spesies ini sebagai langkah awal untuk merevisi taksonomi dan untuk mendapatkan lebih banyak informasi tentang status konservasi spesies ini.

2.1 Introduction

Southeast Asia is one of the richest biodiversity regions on earth. Its complex geological history contributed to unique biota and high concentration of endemic species (Myers et al., 2000). The region also suffered the highest rate of habitat loss and associated biodiversity due to deforestation and global warming (Carr, 2004; Sodhi and Brook, 2006; Sodhi et al., 2010). More than 7 million hectares of forests were lost per year between 2000 and 2010 to meet rising demands for food, fuel and fibers (FAO, 2016). Global warming accelerates the current biodiversity crisis that will especially lead to the extinction of those species living on mountain tops (Spehn et al., 2010). To measure the impact of deforestation and global warming on the species level, biodiversity indicators are very useful to monitor local biodiversity losses (Caro and O'Doherty, 1999; Lindenmayer, Margules and Botkin, 2000; Soberón, Rodríguez and Vázquez-Domínguez, 2000; Kati et al., 2004). Orchids are an ideal flagship group to investigate biodiversity changes because of their enormous popularity amongst plant enthusiasts worldwide and widespread distribution (Newman et al., 2007). Unfortunately, the number of professional orchid taxonomists is dwindling. On the other hand, wildlife photography has been on the rise in the last decades because of the improved technology of cameras, lower costs and more accessible web-based portals to store and share photographs online. Free web-based portals such as Pbase, Facebook, Flicker, Pinterest, Instagram, Google+, SmugMug and other sites provide a platform to exchange photographs accessible to anyone, anywhere and anytime. Portals such as Google+, Yahoo's Flicker and SmugMug also provide features like geotagging, enabling users to add additional data such as when and where a photograph was made in the field

The downside of photographs uploaded by orchid enthusiasts is that plants are often incompletely or wrongly identified, especially when it concerns orchids for which no comprehensive, up-to-date taxonomic information is available. To correctly identify a plant species, plants need to be keyed out with the help of field guides, containing a description or key of all species known to occur in an area. Plant identification can be challenging, especially for novices when they have to use dichotomous keys filled with specialistic terms (Mangold and Parkinson, 2013). With the onset of the internet era, online and real-time information can

be shared, including interactive online keys for species identification. Several software packages for making interactive online keys by converting paper-printed dichotomous keys into computer-aided interactive keys are already available such as LINNAEUS 2.0, Lucid or FRIDA (Farr, 2006; Lindsay and Middleton, 2009; Martellos, 2010). For Orchidaceae, online identification keys have already been developed for species of European orchids and the genera *Cypripedium* L. and *Vanda* Jones ex R.Br. using the Lucid3 and Xper3 platforms at the University of Basel (https://orchid.unibas.ch/index.php/en/orchidinfos/orchid-keys) The interactive keys produced with these programs are much more user-friendly than traditional keys. They can therefore be used by a broad range of users ranging from novice plant enthusiasts up to professionals. With more accessible identification keys, it will become increasingly easy for novice users to identify plants photographed in the wild correctly.

Better identification of plants in the wild is especially needed for overlooked taxa. A prime example of such are the necklace orchids (Coelogyninae), a popular group often seen in cultivation because of their showy flowers. They belong to the subfamily Epidendroideae and comprise a total of 16 genera (Gravendeel et al., 2001; Gravendeel, de Vogel and Schuiteman, 2005; Kosina and Szkudlarek, 2015). *Glomera* Blume is one of the least known genera of the necklace orchids. Species of this genus are rarely cultivated. When not in flower, most species resemble a small ericaceous shrub rather than an orchid and, regarding biomass, *Glomera* is one of the predominant orchid genera in the montane forests of New Guinea (www.orchids.naturalis.nl). A total of 169 species are known of *Glomera* after 27 species of *Glossorhyncha* Ridl. were united under *Glomera* in 2016 (Shaw, 2016; Govaerts et al., 2018). The key characteristics of the genus are the elongated, often branching stem with many leaves that are enveloped by warty sheaths at the base. These species mainly occur in New Guinea but some have expanded their distribution up to Fiji, the Philippines and the New Hebrides. Most are epiphytes or terrestrials in either lowland or montane rainforest up to subalpine environments. Flowers are mostly white, but some species have orange, salmon-pink or green-colored flowers. Inflorescences are usually single-flowered, but some species have multiple flowered inflorescences.

Traditional keys that already exist for *Glomera* are in the English and German languages only and either restricted to genus level or specific geographical

regions. Examples include keys for Fiji (Kores, 1989) and Papua New Guinea (van Royen, 1979; www.orchids.naturalis.nl). With this publication, we present an up-to-date and accessible multilingual interactive key using the Linnaeus NG platform for identification of all species of *Glomera* in Southeast Asia.

Table 2.1. The 169 species of *Glomera* and their distributions included in the keys in alphabetical order.

Species	Distributions
Glomera acicularis Schltr.	Papua New Guinea
G. acuminata J.J.Sm	Indonesia, Papua New Guinea
G. acutiflora (Schltr.) J.J.Sm.	Papua New Guinea
G. adenandroides (Schltr.) J.J.Sm.	Papua New Guinea
G. adenocarpa (Schltr.) J.J.Sm.	Indonesia, Papua New Guinea
G. affinis J.J.Sm.	Indonesia, Papua New Guinea
G. albiviridis P.Royen	Indonesia, Papua New Guinea
G. altigena (P.Royen) J.M.H.Shaw	Papua New Guinea
G. altomontana (Gilli) J.M.H. Shaw	Papua New Guinea
G. amboinensis (Ridl.) J.J.Sm.	Indonesia, Papua New Guinea, Bismarck Islands
G. ambricaulis (P.Royen) J.M.H. Shaw	Papua New Guinea
G. ambuensis (P.Royen) J.M.H. Shaw	Papua New Guinea
G. angiensis J.J.Sm.	Indonesia
G. antaresensis (P.Royen) J.M.H. Shaw	Indonesia, Papua New Guinea
G. appendiculoides Ormerod.	Papua New Guinea
G. asperata Schltr.	Papua New Guinea
G. aurea Schltr.	Indonesia, Papua New Guinea
G. bambusiformis Schltr.	Papua New Guinea
G. bismarckiensis J.J.Sm.	Papua New Guinea
G. bougainvilleana Ormerod	Papua New Guinea
G. brachychaete (Schltr.) J.J.Sm.	Papua New Guinea
G. brassii Ormerod.	Papua New Guinea
G. brevipetala J.J.Sm.	Indonesia, Papua New Guinea

G. caespitosa (P.Royen) J.M.H. Shaw	Papua New Guinea
G. calocephala Schltr.	Papua New Guinea
G. carnea J.J.Sm.	Indonesia
G. carolinensis L.O. Williams	Republic of Kiribati
G. celebica (Schltr.) J.J.Sm.	Indonesia
G. chlorantha (P.Royen) J.M.H.Shaw	Papua New Guinea
G. compressa J.J.Sm.	Papua New Guinea
G. confusa J.J.Sm.	Papua New Guinea
G. conglutinata J.J.Sm.	Indonesia
G. crispa (P.Royen) J.M.H.Shaw	Indonesia
G. cristata (P.Royen) J.M.H.Shaw	Papua New Guinea
G. cyatheicola P.Royen	Papua New Guinea
G. dekockii J.J.Sm.	Papua New Guinea
G. dentifera J.J.Sm.	Indonesia
G. dependens (Schltr.) J.J.Sm.	Papua New Guinea
G. diffusa (P.Royen) J.M.H.Shaw	Indonesia
G. diosmoides (Schltr.) J.J. Sm.	Papua New Guinea
G. dischorensis (Schltr.) J.J.Sm.	Papua New Guinea
G. distichifolia Ormerod	Vanuatu
G. dubia J.J.Sm.	Indonesia
G. elegantula (Schltr.) J.J.Sm.	Indonesia, Papua New Guinea
G. emarginata Kores	Fiji
G. ericifolia Ridl.	Indonesia
G. erythrosma Blume	Indonesia
G. flaccida (Schltr.) J.J.Sm.	Papua New Guinea
G. flamulla Schltr.	Papua New Guinea
G. fluviatilis (P.Royen) J.M.H.Shaw	Papua New Guinea
G. fransseniana J.J.Sm.	Indonesia
G. fruticula J.J.Sm.	Papua New Guinea
G. fruticulosa Schltr.	Papua New Guinea
G. fusca Schltr.	Papua New Guinea
G. fuscosetosa Schuit. & de Vogel	Papua New Guinea
G. gamosepalata P.Royen	Indonesia
G. geelvinkensis J.J.Sm.	Indonesia

G. geminata Ormerod.	Indonesia
G. glomeroides (Schltr.) J.J.Sm.	Papua New Guinea
G. goliathensis J.J.Sm.	Indonesia
G. graminifolia Schltr.	Papua New Guinea
G. grandiflora J.J.Sm.	Indonesia
G. grandilabella (P.Royen) J.M.H.Shaw	Papua New Guinea
G. hamadryas (Schltr.) J.J.Sm.	Indonesia, Papua New Guinea
G. hubrechtiana J.J.Sm.	Indonesia
G. hunsteiniana (Schltr.) J.J.Sm.	Papua New Guinea
G. imitans (Schltr.) J.J.Sm.	Papua New Guinea
G. inconspicua J.J.Sm.	Indonesia, Papua New Guinea
G. inflata (Schltr.) J.J.Sm.	Papua New Guinea
G. jabiensis J.J.Sm.	Indonesia
G. kamay-nolomi Ormerod.	Papua New Guinea
G. kaniensis Schltr.	Papua New Guinea
G. kanke P.Royen	Indonesia, Papua New Guinea
G. kerewensis (P.Royen) J.M.H.Shaw	Papua New Guinea
G. keysseri (Schltr.) J.M.H.Shaw	Papua New Guinea
G. keytsiana J.J.Sm.	Indonesia
G. kuperensis Ormerod.	Papua New Guinea
G. lancipetala J.J.Sm.	Indonesia
G. latilinguis J.J.Sm.	Indonesia
G. latipetala (Schltr.) J.J.Sm.	Papua New Guinea
G. ledermannii (Schltr.) J.J.Sm.	Papua New Guinea
G. leucomela (Schltr.) J.J.Sm.	Papua New Guinea
G. longa (Schltr.) J.J.Sm.	Papua New Guinea
G. longicaulis J.J.Sm.	Indonesia, Papua New Guinea
G. macdonaldii (Schltr.) J.J.Sm.	Papua New Guinea, New Caledonia, New Hebrides, Fiji
G. macrantha J.J.Sm.	Papua New Guinea
G. macrophylla Schltr.	Papua New Guinea
G. manicata J.J.Sm.	Papua New Guinea
G. mayuensis Ormerod	Papua New Guinea

G. melanocaulon Schltr.	Papua New Guinea
G. merrillii Ames	The Philippines
G. microphylla J.J.Sm.	Indonesia
G. minjensis (P.Royen) J.M.H.Shaw	Papua New Guinea
G. minutigibba J.J.Sm.	Indonesia
G. montana Rchb.f.	Papua New Guinea, Solomon, Fiji, Samoa, Vanuatu
G. monticuprina (P.Royen) J.M.H.Shaw	Indonesia
G. muscicola (P.Royen) J.M.H.Shaw	Indonesia
G. myrtillus (Schltr.) Schuit. & de Vogel	Papua New Guinea
G. nana (Schltr.) J.J.Sm.	Papua New Guinea
G. neohibernica Schltr.	Papua New Guinea
G. nigricans (P.Royen) J.M.H.Shaw	Papua New Guinea
G. nigrilimbata P. Royen	Papua New Guinea
G. nigrimarginata (P.Royen) J.M.H.Shaw	Papua New Guinea
G. noroma (P.Royen) J.M.H.Shaw	Papua New Guinea
G. obovata (Schltr.) J.J.Sm.	Papua New Guinea
G. obtusa Schltr.	Papua New Guinea
G. oligantha Schltr.	Indonesia
G. palustris J.J.Sm.	Indonesia, Papua New Guinea, Vanuatu, Solomon
G. palustris var. subintegra J.J.Sm.	Papua New Guinea
G. papuana Rolfe	Papua New Guinea
G. parviflora J.J.Sm.	Indonesia
G. patens Schltr.	Papua New Guinea
G. pendulosa J.M.H.Shaw	Papua New Guinea
G. pensilis (Schltr.) J.J.Sm.	Papua New Guinea
G. pilifera (Schltr.) J.J.Sm.	Papua New Guinea
G. pinifolia (P.Royen) J.M.H. Shaw	Indonesia
G. platypetala Schltr.	Indonesia
G. pleiotricha J.J.Sm.	Indonesia, Papua New Guinea

G. plumosa J.J.Sm.	Indonesia, Papua New Guinea
G. polychaete (Schltr.) J.J.Sm.	Papua New Guinea
G. pseudomonanthos Ormerod	Indonesia, Papua New Guinea
G. pteropetala (Schltr.) J.J.Sm.	Papua New Guinea
G. pullei J.J.Sm.	Indonesia, Papua New Guinea
G. pumilio J.J.Sm.	Indonesia
G. pungens (Schltr.) J.J.Sm.	Papua New Guinea
G. retusa J.J.Sm.	Indonesia
G. retusimentum J.J.Sm.	Indonesia
G. rhombea J.J.Sm.	Indonesia
G. rigidula J.J.Sm.	Papua New Guinea
G. rubroviridis J.J.Sm.	Indonesia
G. saccharipanis Ormerod.	Papua New Guinea
G. saccosepala J.J.Sm.	Papua New Guinea
G. salicornioides J.J.Sm.	Indonesia
G. salmonea J.J.Sm.	Indonesia, Papua New Guinea
G. sandaveri Ormerod.	Papua New Guinea
G. scandens J.J.Sm.	Indonesia
G. schlechteriana Mansf.	Papua New Guinea
G. schultzei Schltr.	Papua New Guinea
G. scopulata (P. Royen) J.M.H. Shaw	Indonesia
G. secunda J.J.Sm.	Indonesia
G. sepalosiphon Schuit. & de Vogel	Papua New Guinea
G. similis J.J.Sm.	Indonesia
G. sororia J.J.Sm.	Indonesia
G. squamulosa (Schltr.) J.J.Sm.	Papua New Guinea
G. stenocentron (Schltr.) J.J.Sm.	Indonesia, Papua New Guinea
G. stolonifera Ormerod	Papua New Guinea
G. subeciliata J.J.Sm.	Indonesia
G. sublaevis J.J.Sm.	Indonesia
G. subnivalis J.M.H.Shaw	Indonesia
G. subpetiolata Schltr.	Papua New Guinea
G. subracemosa J.J.Sm.	Indonesia, Papua New Guinea
G. subulata (Schltr.) J.J.Sm.	Papua New Guinea

G. subuliformis J.J.Sm.	Indonesia
G. tamiana J.J.Sm.	Papua New Guinea
G. tenuis (Rolfe) J.J.Sm.	Papua New Guinea
G. terrestris J.J.Sm.	Indonesia, Papua New Guinea
G. torricellensis Schltr.	Papua New Guinea
G. tortuosa (P.Royen) J.M.H.Shaw	Papua New Guinea
G. transitoria J.J.Sm.	Indonesia
G. triangularis J.J.Sm.	Papua New Guinea
G. tubisepala (P.Royen) J.M.H.Shaw	Papua New Guinea
G. umbrosa P.Royen	Indonesia
G. uniflora J.J.Sm.	Indonesia
G. verrucifera Schltr.	Papua New Guinea
G. verrucosissima (Schltr.) J.J.Sm.	Papua New Guinea
G. verruculosa (Schltr.) J.J.Sm.	Papua New Guinea
G. versteegii J.J.Sm.	Indonesia, Papua New Guinea
G. viridis (Schltr.) J.J.Sm.	Papua New Guinea

2.2 Software technical specification

Linnaeus NG (http://linnaeus.naturalis.nl/) is a web-based species information management system. Linnaeus NG has several modules such as species and additional features such as media (in which distribution maps and illustrations can be found) and two types of keys. For this study, a single-entry key and multi-entry key were built. Linnaeus NG has been developed using open source techniques (PHP, MySQL) and is hosted in a Linux environment. On the client-side, project administrators interact with the program through a web browser. A recent version of all major browsers is supported for regular platforms and tablets. Currently, Linnaeus NG is proprietary software; updates and changes can only be made in agreement with the Naturalis Biodiversity Center. However, access to Linnaeus NG is not limited to employees or associates of Naturalis Biodiversity Center and can be granted on request.

Table 2.2. Morphological characters and their states used in the keys.

Plant part	Character	States
Rhizome	Division	Heavily branched; not or only sparsely branched
Leaf blade	Color	Green; reddish-brown
	Lamina	Fleshy; not fleshy
	Dots	With brown dots; without brown dots
		One lobe (obtuse or acute);
	Tip	two lobes (acute-acute, obtuse-obtuse or acute-obtuse)
Leaf sheath	Color	Green; black
	Tooth	With tooth; without tooth
	Notch	Notched; not notched
	Bristles	With bristles; without bristles
	Warts	With warts; without warts
Spathe	Warts	With warts; without warts
	Hairs	With hairs; without hairs
	Dots	With brown dots; without brown dots
Floral bract	Warts	With warts; without warts
	Hairs	With hairs; without hairs
	Dots	With brown dots; without brown dots
	Size	Longer than spathe; shorter than spathe
Inflorescence	Number of flowers	One; more than one
Flower	Color	White; green; pinkish-salmon; orange; red

Orientation	Upright; up-side-down
Spur length	Shorter than 10 mm; longer than 10 mm
Spur tip	One-lobed; two-lobed
Lateral sepals	Free; fused for more than two-thirds
Lip	With glands on tip; without glands on tip
Lip tip color	White; black; green; red; grey; pink
Odor	Fragrant; not fragrant
Sepal orientation	Straight; bent backward
Column foot	Present; absent
Ovary ribs	With ribs; without ribs
Ovary dots	With brown dots; without brown dots
Ovary warts	With warts; without warts

2.2.1 Conditions of use

Linnaeus NG version 2.5 is free to use for personal and non-commercial use. Data will be guaranteed for long term sustainable hosting if they are complying with national standards in research and education. The data must be sharable and free to access and, in later stages, the developer may include adequately licensed content in Bioportal (http://bioportal.naturalis.nl/).

2.2.3 User interface

Users can access the key at https://glomera.linnaeus.naturalis.nl/ and it can be used online using any web browser. No additional software is required. The interface was designed to be able to access from any device with flexible layout. The navigation menu is shown on the left side. The menu includes an index,

Table 2.3. Non-morphological characters and their states used in the keys.

Character group	Character	States
Ecology	Lifeform	Epiphyte; terrestrial
Flowering season	Months	January; February; March; April; May; June; July; August; September; October; November; December
Global distribution	Country	Indonesia; Papua New Guinea; Fiji; New Hebrides; Philippines
Distribution in Indonesia	Island	Papua; Java; Moluccas; Sulawesi
Occurrence over the elevational gradient	Altitude	Lowland; midland; highland

species list, single-access key, multi-access key, two language options (English or Indonesian) and a glossary. A user can directly search for a species by using the search box on the top. If the user does not yet have a clue about the identity of the species, a single-access key is available with 166 steps to help with the identification process. A multi-access key is also provided, in which remaining choices with 100% fit only are indicated at every step. A glossary is present to help novice users to understand terms used in the descriptions and keys.

2.3 Data

Morphological characters used in the interactive key (Figure 2.1; Table 2.2) were initially selected from already existing dichotomous keys. Not all characters turned out to be clear to both advanced and novice users, though, so the number was reduced to a final selection after tests of preliminary versions of the key by students of the annual Orchid Biology Course taught at Basel University in 2017. Students were provided with a similar set of specimens and we monitored if they were able to come up with the correction identification within half an hour and

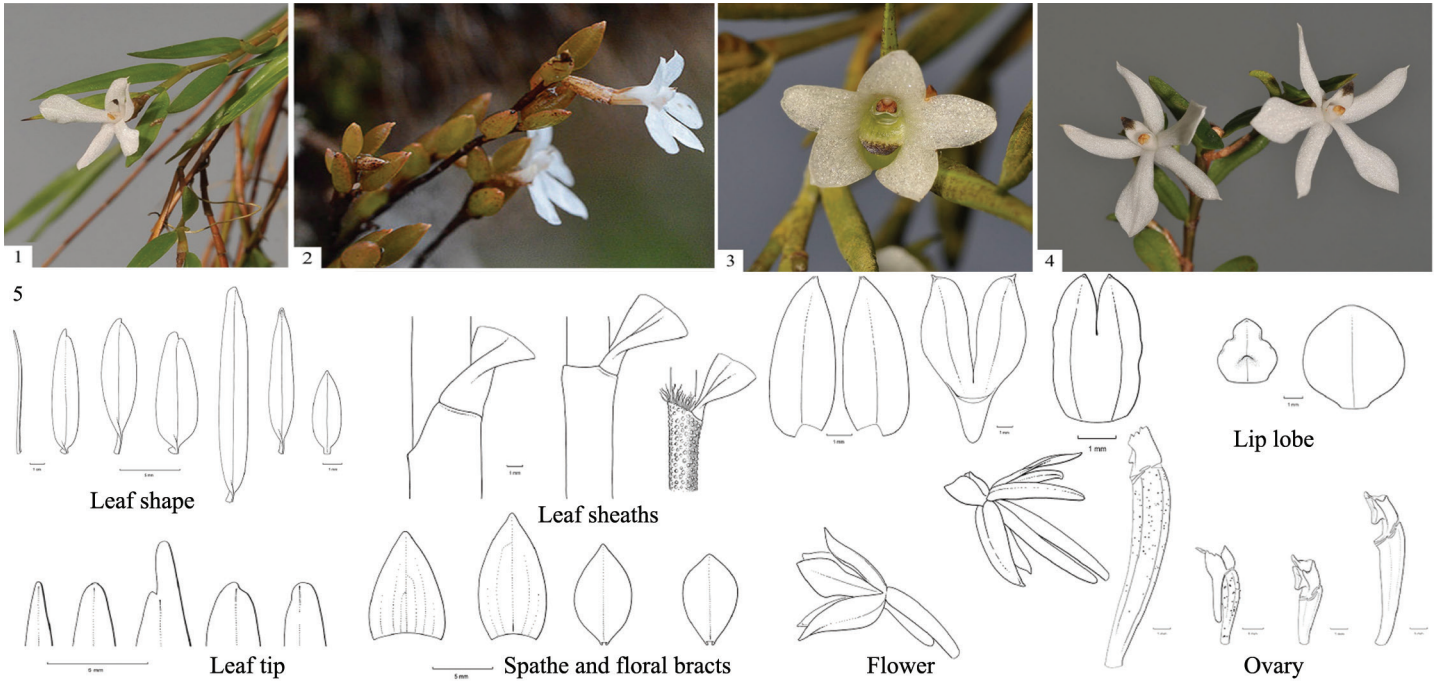

Figure 2.1. Illustrations of a selection of key characters used in the identification keys. 1. *Glomera acutiflora* (Schltr.) J.J.Sm. with green leaves (photograph by Rogier van Vugt). 2. *Glomera sp.* with reddish-brown leaves (photograph by fotosynthesys deposited on FLICKR). 3. *Glomera pungens* (Schltr.) J.J.Sm. with up- right flowers (photograph by Rogier van Vugt). 4. *Glomera hamadryas* (Schltr.) J.J.Sm. with flowers turned up-side-down (photograph by Rogier van Vugt). 5. Various shapes of the leaf blade, leaf tip, leaf sheath, leaf spathe, floral bract, entire flower, sepals, petals, lip and ovary present in *Glomera* and *Glossorhyncha* (illustrations by Esmée Winkel).

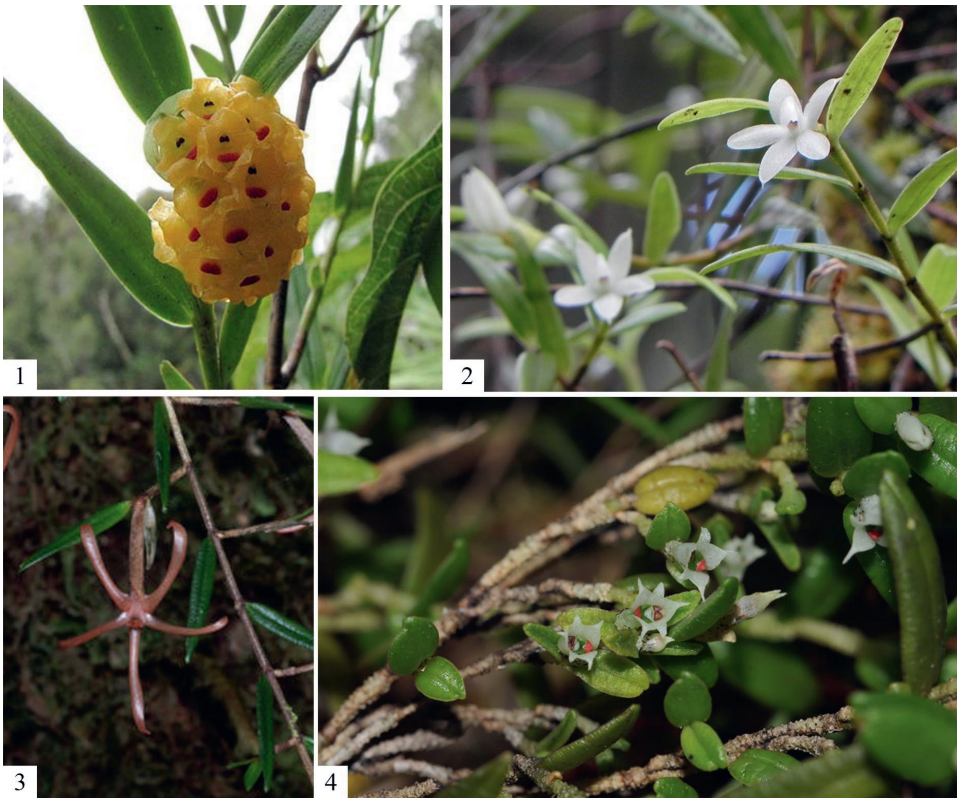

Figure 2.2. Photographs of *Glomera* species collected from online platforms. 1. *Glomera aurea* (photograph by Mehd Halaouate). 2. *Glomera macdonaldii* (photograph by Benoit Henry). 3. *Glomera tubisepala* (photograph by Gary Yong Gee). 4. *Glomera glomeroides* (photograph by S.A. James).

which characters were considered easy to use and which were not. Both keys were constructed independently. For the single-entry key, it is possible to start at any step and, if you want to start again, you have to click on step 1. For the multi-entry key, you have to select the option 'Start all over' if you want to start again. All character states used are illustrated by images. Apart from morphology, geographical distribution and ecology can be informative as well. The key therefore also includes a few of these non-morphological characters (Table 2.3).

2.4 Discussion

The interactive key presented here for *Glomera* of Southeast Asia encompasses more species and geographic areas than any existing key currently available for this genus. Next to English, it was also written in a language commonly used in Southeast Asia, Bahasa Indonesia, which enables a much wider group consisting of both novice and advanced users in the region to identify these orchids correctly. The main challenges to construct this key consisted of the fact that type descriptions were often rather vague and that many type collections were lost after the bombing of the herbarium of the Botanic Garden and Botanical Museum in Berlin in the second world war. Of the 169 species, a total of 52 types were lost. We therefore studied a lot of additional collections, all listed under Species, option Collection specimens on the website, to verify character states.

Compared with traditional dichotomous keys, interactive keys can be used much more easily by relatively novice users (Jacquemart et al., 2016). Users of this key can quickly survey images of remaining species after selection of a first set of characters such as flower color and distribution. This key therefore enables much faster (i.e. seconds rather than minutes) identification of the best candidate from the remaining choice than working through a traditional key until the final result appears with little indication of the remaining potential outcomes during the identification process. When using a conventional dichotomous key, a user is not provided with an indication of the remaining taxa during the identification process and can only hope that the final outcome matches the candidate. Professional taxonomists often rely on extensive previous exposure to species during the identification process but novice users cannot fall back on this.

Our key will hopefully urge the users to further enrich the database and help update the distribution of species or detect possible new species. Pictures on the web placed there by enthusiasts are considered to be an essential source for the discovery of new data (Marshall, 2018). Use of our key by more wildlife photographers will help to record the presence of species in geographic regions where they were previously overlooked. The idea of democratisation of taxonomy by involving the general public (hobbyists, naturalists, tourists) to this field could trigger higher interest in currently unexplored taxa. Recently, the taxonomy and biogeography of diving beetles in Bali could, for instance, be improved by using

citizen scientists and social networks such as Facebook and WhatsApp (Suprayitno et al., 2017).

A first indication that the same might happen for *Glomera* orchids is illustrated by the fact that we could combine historical, literature-based data and recent photographs of plants taken by wildlife photographers of *G. aurea* Schltr., *G. macdonaldii* (Schltr.) J.J. Sm., *G. tubisepala* (P. Royen) J.M.H. Shaw and *G. glomeroides* Schltr. Pictures of flowering plants, taken in Papua of the first species, the Solomon island of the second and Papua New Guinea of the third and the fourth and deposited on Flickr and Smug- Mug, came to our attention during this study. Once contacted by us, the photographers provided more detailed locality data and dates, which enabled us to update the distribution maps and also the flowering time of these species.

We also used our key to assign a name to a yet unknown *Glomera* species photographed. For example, on the photograph of *Glomera* sp. 2010-064 uploaded in the username PNG Collection of Smugmug, we could see details such as the shape of the leaf sheath, tooth and warts, color and shape of the leaves and leaf apex and the color of the flower (white with a green lip and red lip tip). After selecting all the characters that could be identified from the photograph and additional data such as location and altitude, we could reduce the number of species from 169 to 10. We ended up with identifying it as cf. *G. glomeroides* by comparing the photograph to a drawing made by Friedrich Richard Rudolf Schlechter in 1923 that accompanied the type description. The type of this species was lost in the herbarium of Berlin and no documented photograph has yet been published. The photographer could provide us with locality data in the Madang province of Papua New Guinea. With the aid of our interactive key, we could therefore simplify the process of identification of this unidentified species.

2.5 Conclusions

We expect that the interactive key presented here for *Glomera* of Southeast Asia will enable a higher number of people to collect more precise and more reliably identified observations of species of this overlooked orchid genus. It is user-friendly due to the many illustrations and color photographs, encompassing a combination of historical, literature-based and recent, web-mined data of all species rather than

subsets only and written in a language commonly spoken in parts of the world where these orchids occur in the wild. The key was designed for efficient use by both inexperienced and advanced orchidologists. This publication accompanies the release of version 1.0. We encourage all users to provide feedback to improve and further expand this version by contacting us by email to gain more insight into the current distribution of these overlooked orchids. This will enable us to accurately assess their conservation status. By obtaining more knowledge of the regions of distributions but without disclosing too detailed locality data, we hope to prevent extinction of these orchids in the wild.

Chapter 3

Identification of sterile orchids in living collections by DNA barcoding of types

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Abstract. Orchid collections in botanic gardens are an essential source for biodiversity research. Most species require very specific temperature, humidity, light levels and nutrient concentrations for flower induction and survival and therefore often remain sterile or die shortly after collection from the wild. This severely hampers the identification of such collections to species level. DNA barcodes obtained from fertile type specimens in herbaria are a potential tool for fast species identification of sterile living collections, but only a few curators of herbaria allow destructive sampling of type specimens. We obtained permission to perform destructive DNA extraction of a small number of the numerous leaves from a total of 32 type and 11 non-type specimens of the poorly known necklace orchid genus *Glomera* Blume preserved in the herbarium of Naturalis Biodiversity Center (L) and Herbarium Bogoriense (BO) that were collected between 5 up to 194 years ago. We used four primer combinations to fully sequence the nrITS region of these specimens and fresh sterile living collections and obtained Sanger sequences for 38 dried specimens and 43 living collections with a length varying between 70-887 bp. A possible correlation between age and length of DNA barcodes retrieved was investigated. With the short sequences obtained, a total of 6 sterile living collections could be identified to species level. No correlation between age and relative length of DNA barcodes retrieved was found. A total of 38 living collections remained unnamed and are possibly new to science. Our result shows that DNA barcodes obtained from type material can provide reliable taxonomic information of sterile living collections. We propose a less rigorous policy regarding permission to generate DNA barcodes from type specimens to improve the identification of sterile specimens in living collections for better protection of poorly known orchid genera.

3.1 Introduction

Many countries have a different strategy for plant conservation that can either take place in situ, ex situ or both, and at species and/or population level. Plant conservation supports the recovery and reintroduction of endangered species by developing a gene bank and/or restoration ecology (Heywood, 2017). A botanic garden is the equivalent of a museum of living plants where species can be studied, not only by experts to increase scientific knowledge, but also by general plant enthusiasts for educational and recreational purposes (Holttum, 1970). Orchidaceae are one of the most diverse angiosperm families with more than 25,000 species (Dressler, 1993). Because of their spectacular diversity in life forms and flowers, orchids are usually present in collections of botanic gardens. For conservation purposes, many orchids have been collected from different locations and habitats. These orchid collections have been important sources for studies on conservation, ecology, evolution, germination, physiology, pollination and taxonomy. Many new orchid taxa have been described from specimens in collections of botanic gardens. Especially tropical orchids have highly diverse flowers that evolved due to highly specific plant-pollinator interactions (Kim et al., 2014). In greenhouses, these collections are kept alive, but due to the highly diverse requirements of temperature, light levels and nutrition for each species, it is a challenge to stimulate flowering (Wang, 2000; Lopez and Runkle, 2004, 2005; Vaz et al., 2004; Pfeifer et al., 2006). Some accessions therefore remain sterile for their entire life, or die shortly after collection from the wild. Since it is extremely difficult or impossible to identify tropical orchids lacking flowers (Cameron et al., 1999), many specimens in living collections therefore remain unnamed.

Accurate identification of newly discovered species and a formal description are essential for plant conservation (Lahaye et al., 2008; Gutiérrez, 2010; Strutzenberger et al., 2012). Due to extensive variation of morphological characters within a single species, descriptions of new species solely based on morphological characters are not published that much anymore (Bogarín et al., 2018) especially when it comes down to the lesser-known orchid genera. DNA barcoding is an established tool for rapid identification of a species with a short

standard DNA sequence (Hebert et al., 2003). Fresh tissue is usually harvested to obtain DNA sequences of high quality.

When fresh material is not available, dried material may be tried. Several studies showed the use of herbarium material as an underutilised genomic treasure (Blattner, 1999; Eloff, 1999; Särkinen et al., 2012; Bakker et al., 2016; Xu et al., 2015; Hart et al., 2016). Especially for type specimens, most herbarium curators do not allow destructive sampling, even if only a small part of the type is requested. Arguments used are that destructive sampling for DNA extraction might cause irreparable damage to the specimens, which conflicts with their historical and scientific importance (Staats et al., 2011).

In this study, we focused on *Glomera* Blume, an underexplored genus from subtribe Coelogyninae. *Glomera* currently comprises 169 species that are widely distributed in Indonesia, Papua New Guinea, The Philippines, Bismarck Archipelago, Solomon, New Caledonia, and Fiji (Wati, van Vugt and Gravendeel, 2018). We studied the living *Glomera* collections in the greenhouse of the Hortus botanicus Leiden and Bali Botanic Gardens, where many specimens remained unidentified because they never flowered in cultivation. Since these orchids have many small leaflets, we obtained permission from the curators of the herbaria of Naturalis Biodiversity Center in the Netherlands and Herbarium Bogoriense in Indonesia to generate DNA barcodes of type specimens to identify sterile orchids in collection of the Hortus botanicus and Bali Botanic Gardens.

3.2 Material and Methods

3.2.1 Taxon sampling

We sampled a total of 81 *Glomera* specimens. A total of 43 fresh leaf fragments were collected from living plants in the orchid greenhouses of the Hortus botanicus Leiden, The Netherlands, and Bali Botanic Gardens, Indonesia. A total of 38 leaf samples were collected from dried herbarium specimens present in the herbaria of Leiden (L) and Bogor (BO), where most types of the genus *Glomera* are deposited. Of the 38 herbarium samples analysed, 24 were type specimens, belonging to species collected in the same geographical regions as the orchids from the living collections. Only a small piece of leaf tissue (1 cm2) was collected. The specimen age ranged from 5 to 194 years (see Table 3.1).

Table 3.1. Details of herbarium specimens analyzed in this study.

Species	Specimen voucher	Specimen collection year
G. acutiflora (Schltr.) J.J.Sm.	L0061314	1906
G. acutiflora (Schltr.) J.J.Sm.	L1521087	2003
G. amboinensis (Ridl.) J.J.Sm.	L0043490	1912
G. diosmoides (Schltr.) J.J.Sm.	L0061263	1965
G. angiensis J.J.Sm.	LSJ 1861	2013
G. bambusiformis Schltr.	L0061317	1908
G. compressa J.J.Sm.	L0056417	1920
G. confusa J.J.Sm.	RK 197	2013
G. erythrosma Blume	L0061325	1825
G. gamosepalata P.Royen	L0063654	1938
G. hamadryas (Schltr.) J.J.Sm.	L0056420	1953
G. hamadryas (Schltr.) J.J.Sm.	L0056422	1913
G. hamadryas (Schltr.) J.J.Sm.	L0056421	1910
G. hamadryas (Schltr.) J.J.Sm.	L0056423	1908
G. hamadryas (Schltr.) J.J.Sm.	L0064472	1908
G. hamadryas (Schltr.) J.J.Sm.	L0056419	1920
G. kanke P.Royen	L0056414	1971
G. kanke P.Royen	L0061331	1961
G. kanke P.Royen	L0064500	1972
G. noroma (P.Royen) J.M.H.Shaw	L 0061278	1961
G. palustris J.J.Sm.	L0056415	1965
G. palustris J.J.Sm.	L0061334	1920
G. pullei J.J.Sm.	L0426043	1913
G. secunda J.J.Sm.	L0043484	1918
G. sp.	LSJ 1885	2016
G. sp.	LSJ 1850	2016
G. sp.	LSJ 1862	2016
G. sp.	Droissart & Juswara 1779	2014
G. sp.	RK 126	2013
G. sp.	RK 18	2013

G. sp.	WB 483	2014
G. sp.	GT 2383	2006
G. sp.	WB 476	2014
G. sp.	LSJ 1854	2016
G. sp.	GT 2894	2010
G. sp.	LSJ 1859	2016
G. sp.	HBL 20111281	2011
G. viridis (Schltr.) J.J.Sm.	LSJ 1882	2016

3.2.2 DNA extraction, amplification and sequencing

Leaf fragments were placed in 2 ml reaction tubes containing a glass bead (7 mm diameter) and homogenized in a Retsch Mixer Mill, MM400 for 3 minutes at 30 Hz/second into a fine powder. Total genomic DNA of herbarium and silica-dried material was extracted using the 2x CTAB (Cetyltrimethylammonium bromide) method (Doyle and Doyle, 1987) with longer DNA precipitation steps for the herbarium samples: these extracts were stored at -20°C for one week to obtain a higher DNA yield. The nuclear ribosomal ITS-5.8S-ITS2 (nrITS) region of silica-gel dried of freshly harvested leaf material was amplified using primers 17SE (5'-ACGAATTCATGGTCCGGTGAAGTGTTC-3') and 26SE (5'-TAGAATTCCCCGGTTCGCTCGCCGTTAC-3') as described by Sulistyo et al. (2015).

Subsequently, a M13 universal sequencing primer was added to the 5' end of the forward (ACGAATTCATGGTCCGGTGAAGTGTTC) and reverse (TA-GAATTCCCCGGTTCGCTCGCCGTTAC) primers to improve Sanger sequencing efficiency. Each PCR reaction had an end volume of 25 µl, containing the template DNA, CoralLoad PCR buffer (Qiagen), dNTPs, Taq DNA Polymerase (Qiagen), and both primers. The PCR reactions were carried out using a C1000 Touch Thermal Cycler (Bio-Rad). The following thermocycling program was used to amplify each gene fragment: 96 °C for 5 min (1 cycle); 96 °C for 30 s, 50 °C for 30 s, 72 °C for 1 min (35 cycle); and then 72 °C for 7 min (1 cycle).

The nrITS region of herbarium preserved leaf material was amplified using primer p3 (5'-GACTCYCGGCAATGGATATCTCG-3') and p4 (5'-CCGCTTATTGATATGCTTAAACTCRGC-3') as described by Cheng et

al. (2016) and primer F1 (5'-CGAGTCTTTGAACGCAAGTTGCG-3') and R1 (5'-GGCCAACGAGACGATAACCC-3'), F2 (5'-CTGCGGAAGGATCAT TGTCGAGAC-3') and R2 (5'-TCAAACCGGCGCAGCTTCG-3'), R3 (5'-CGGCTCTCGCATCGATGAAGAG-3'), F3 (5'-ACCAACAGCAAGG-3') and R4 (5'-GCTTGGAATGCGACCCCAGG-3') that were newly designed for this study. Each PCR reaction had an end volume of 25 µl, containing the template DNA, 5x Phire PCR buffer (ThermoScientific), BSA, dNTPs, Phire Hot Start II DNA Polymerase (ThermoScientific), and both primers. The following thermocycling program was used to amplify each gene fragment: 98 °C for 1 min (1 cycle); 98 °C for 10 s, 50 °C for 10 s, 72 °C for 20 s (40 cycle); and then 72 °C for 1 min (1 cycle).

Sanger sequences were assembled and edited in Geneious ® R8 (Biomatters Ltd., Auckland, New Zealand) (Kearse et al., 2012). The ends of all data sets were trimmed to avoid character misinterpretation. Ambiguous bases were replaced with "N" in the data matrix. DNA sequences were aligned using the MAFFT platform (Multiple Alignment Fast Fourier Transform (Katoh and Standley, 2013) as implemented in Geneious ® R8 with subsequent manual adjustment. Missing data were replaced with "?".

3.2.3 Phylogenetic analysis

To assess whether sequences from nrITS formed species-specific clusters, we conducted distance-analysis using Neighbour-Joining (NJ) analyses and phylogenetic analysis using Maximum Likelihood (ML), Maximum Parsimony, and Bayesian interference (BI) with *Coelogyne fimbriata* Lindl. as outgroup based on earlier studies (Gravendeel et al., 2001) that showed this genus most closely related to the genus *Glomera*. The chosen nucleotide substitution model GTR+G was calculated using the Akaike Information Criterion (AIC) in jModelTest2 v.2.1.6 (Darriba et al., 2015). The analyses were run in the CIPRES Science Gateway v.3.1. (Miller et al., 2010). We performed Bayesian interference analyses with Mr.Bayes v.3.2.6 on XSEDE (Huelsenbeck et al., 2004) with the following parameters: number of runs (nruns=2), number of chains to run (nchains=4), number of generations (ngen=5 x 107), temperature parameter (temp=2) and sampling frequency of 2000, yielding 25000 trees per run. The log files from

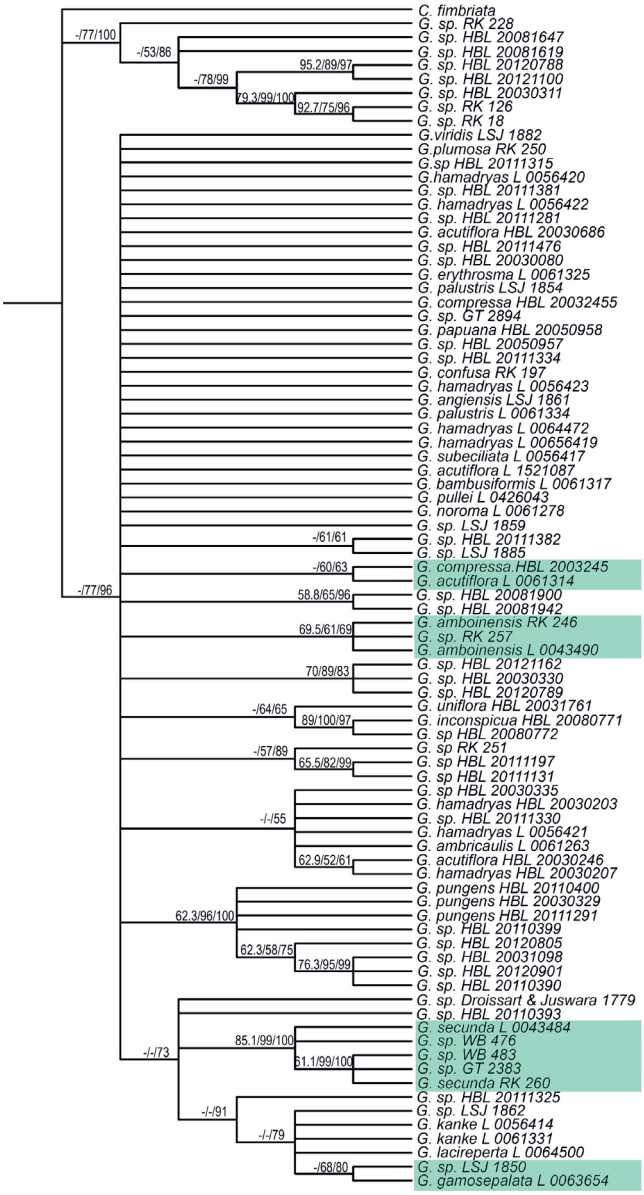

Figure 3.1. Bayesian phylogenetic tree built from aligned sequences from the nrITS region of 81 species and one outgroup. Nodal support values are Neighbour Joining score/bootstrap percentage of Maximum Likelihood/Bayesian posterior probabilities.”-“ indicates bootstrap value <50%. Green colored clades contain herbarium specimens analyzed in this study.

MrBayes were inspected in Tracer v.1.6 to check for convergence of independent runs (i.e., with estimated sample size (ESS)>200).

3.4 Results

3.4.1 Alignment and sequence characteristics

Double-stranded amplifications and complete sequences of nrITS were obtained from 43 fresh specimens of *Glomera*. A total of 38 incomplete sequences were obtained from herbarium specimens. The length of the nrITS sequences varied between 70-887 bp (see Figure 3.2). The oldest specimen investigated was a 195 years old type specimen of *Glomera erythrosma* Blume, from which a 410 bp sequence was recovered, while the youngest was a 4 years old specimen of *Glomera* sp., from which a 404 bp sequence was generated. The length of the DNA sequences obtained from fresh specimens ranged from 706-852 bp (see Figure 3.3).

3.4.2 Phylogenetic analysis

Trees obtained by NJ, ML and Bayesian analyses showed congruent topologies. Figure 3.1 shows the Bayesian tree. Four clades containing sequences obtained from herbarium type specimens were highly supported (>50%) in all three analyses. By matching DNA barcodes, we were able to identify sterile living collections to the following four species of *Glomera*: *G. acutiflora* (Schltr.) J.J.Sm., *G. amboinensis* (Ridl.) J.J.Sm., *G. gamosepalata* P. Royen, and *G. secunda* J.J.Sm. This added

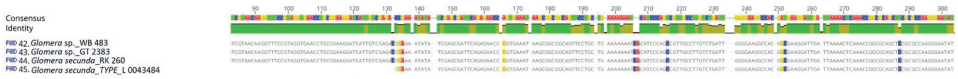

Figure 3.2. Part of the sequence alignment of the nrITS region for different specimens of *G. secunda* J.J.Sm. analysed. The colored nucleotides indicate diagnostic basepairs for this particular species.

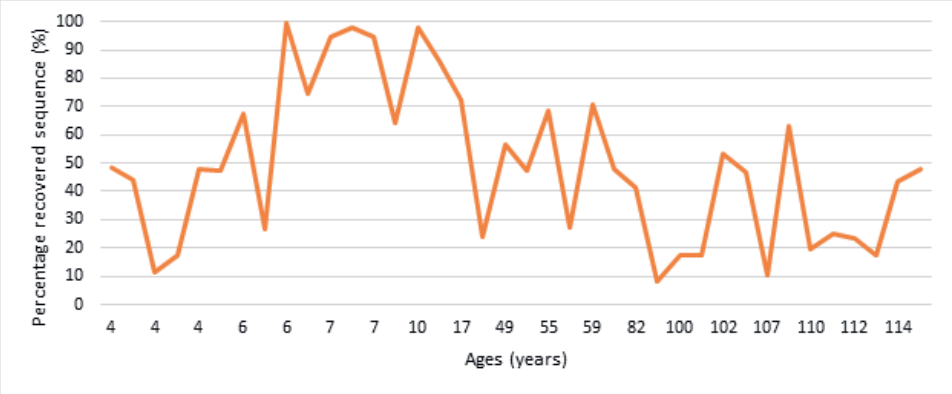

Figure 3.3. Relative recovery of nrITS DNA barcode sequence (%) from herbarium specimens investigated in this study plotted against specimen age (years).

new locality data to their known distributions.

3.5 Discussion

Traditionally, orchid identification heavily relied on morphological characters, both vegetative and floral (Haider et al., 2010). Some of the vegetative (habitus, size, leaf shape) and fertile characters (flower color and size) of *Glomera* species are depicted in Figure 3.4. It is challenging to identify sterile plants to species level as traditional identification keys usually focus on flowering material (Ramalho et al., 2018). DNA barcoding offers a solution to provide rapid and accurate identification using short standardized gene regions (Kress et al., 2005). We show that for the orchid genus *Glomera*, DNA barcoding can be a solution for sterile plant identification. However, not all DNA sequences generated from sterile specimens could be matched with those of type specimens in this study. The still unidentified life plants might belong to undescribed species but could not yet be formally published because flowers are needed for this.

In this study, we show that only small amounts of leaf material (1 cm2) of herbarium specimens were sufficient to generate DNA barcodes, which were sufficiently long and informative to identify sterile specimens in living orchid collections. Especially with species that have many leaves, such as *Glomera*, the sampling of dried specimens can be kept to a minimum. The percentage of recovered DNA sequences was highly variably and no correlation with the age

Figure 3.4. Vegetative and floral characters of *Glomera*. Left: *Glomera inconspicua* J.J.Sm. (HBL 20080772), photograph by Richa Kusuma Wati. Right: *Glomera confusa* J.J.Sm. (HBL 20030288), photograph by Rogier van Vugt.

of the specimen was found. The degradation of DNA in a herbarium is affected by many factors such as the collector's treatment in the field, how rapidly a plant was dried and the storage condition (Hart et al., 2016). A total of 16 of the herbarium specimens analyzed in this study were provided by the Herbarium Bogoriense. DNA sequences recovered were relatively low despite the fact that these specimens were only collected in the past 10 years. The low coverage could be explained by the common method to preserve specimens by compressing the plant and soak it in ethanol 70% in the field before subsequent drying in an oven. The use of ethanol in the tropics, where the high humidity often prevents efficient and continuous dessication, has a negative effect on plant DNA quality (Staats et al., 2011).

The challenges of working with ancient DNA from herbarium material is that only short fragments can be amplified, due to fragmentation caused by ageing. An increasing number of studies show that DNA barcoding is possible from herbarium specimens though (Bakker et al., 2016; Contreras-Ortiz et al., 2019) by designing new primers, as done in this study, or using Next Generation sequencing. The added value of using herbarium collections is that detailed locality data are available (Xu et al., 2015), saving costs for travel to resample. The entire collection of 3095 herbaria in the world today consist of 387 million specimens,

collected from all over the world, and including many rare and possibly already extinct species. Only a small part of these collections are used for DNA-based research, mainly due to the fact that curators are often reluctant to allow destructive sampling (Särkinen et al., 2012; Thiers, 2019). By opening up these collections for DNA barcoding, reference libraries of DNA sequences from especially types could be a welcome solution to help solving the current taxonomic impediment.

3.6 Conclusions

Despite their often degraded DNA, exploitation of DNA barcodes generated from type specimens seems a promising tool to identify sterile specimens in living orchid collections. Additional sequencing of type specimens for DNA barcoding will increase our knowledge of poorly known orchid genera.

Orchid-snail herbivory interactions

Chapter 4

The effect of orchid leaf ornamentation on snail adhesion

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Abstract. Protective structures in the epidermis are essential for land plants to defend themselves against herbivores. Intriguingly, field studies show that some orchid species are less prone to herbivory than other species. In this study, we investigated the effect of leaf ornamentation of the terrestrial orchids *Orchis mascula* (L.) L. and *Calanthe triplicata* (Willemet) Ames and epiphytic orchids *Dendrochilum pallidiflavens* Blume and *Trichotosia ferox* Blume on consumption and attachment of herbivorous land snails, using histochemistry, feeding and centrifuge experiments. Size and ornamentation of wax layers and density and histochemistry of epicuticular hairs and exudates on the orchid leaves were assessed with light microscopy, scanning electron microscopy and transmission electron microscopy. The possible contribution of epicuticular hairs to palatability was investigated by feeding snails untrimmed and trimmed orchid leaves. Total forces needed to detach two differently shaped snail species, *Subulina octona* and *Pleurodonte isabella*, were measured using a turntable equipped with a synchronized strobe. Snails were placed in two positions, either perpendicular or parallel to the main veins on the orchid leaves and on the adaxial (=upper) or abaxial (=lower) side. The results obtained provided three new insights. First of all, trimming of epicuticular hairs increased the palatability of young leaves of *Calanthe triplicata* (Willemet) Ames significantly. Secondly, a perpendicular or parallel position of the snails to the main veins did significantly affect the attachment performance of the smaller species tested, but only on the leaves of *Orchis mascula* (L.) L. that are protected by a wax layer. Thirdly, snails came off significantly faster on leaf sides covered with a high density of lignin filled epicuticular hairs. Our study highlights the importance of histology in combination with attachment force and feeding experiments for obtaining a better understanding of the defense mechanisms employed by different species of epiphytic and terrestrial orchids to deter herbivorous snails.

4.1 Introduction

The surface of sessile organisms like plants plays a crucial role in environmental interactions (Barthlott et al., 2017). Understanding the ecological and evolutionary interactions of plants and herbivores has been a subject of interest for many decades. Plants evolved different strategies to avoid consumption by herbivores. First of all, they have a physical barrier, either through development of a waxy cuticle, trichomes, spines, raphides or setae (Agrawal et al., 2009; Hanley et al., 2007; He et al., 2011; Konno et al., 2014; Sharma et al., 2009; Wagner et al., 2004). Secondly, they can defend themselves chemically by producing secondary metabolites (Barbehenn and Constabel, 2011; Rani and Jyothsna, 2010; Vandenborre et al., 2011). Thirdly, they often evolved a symbiosis with natural enemies of herbivores such as ants (Fiala and Maschwittz, 1992; Fischer et al., 2002; Gong and Zhang, 2014).

To feed on a plant, herbivores need to attach themselves to a plant's surface (Eigenbrode, 2004). Plant physical defensive structures are often embedded in the epidermal cells (Barthlott et al., 2017). Examples of such protective structures are epicuticular hairs and waxes. Trichomes are hair-like appendages extending from the epidermis that can be straight, spiral or hooked, branched, or unbranched, and glandular or non-glandular (Levin, 1973). Mechanically, trichomes can have both toxic and deterrent effects on herbivore attachment. (Hanley et al., 2007). Trichomes in high densities interfere with the movements of small herbivores on a plant, thus reducing access to the leaf surface (Agrawal et al., 2009). Secondary metabolites secreted by glandular trichomes can be poisonous, repellent or even trap insects, thus forming a combination of structural and chemical defense (Hanley et al., 2007; Sharma et al., 2009). Another structural feature produced by plants is a lipophilic material known as epicuticular wax (EW). The main function of EW is waterproofing the cuticle (Schönherr, 1976), but the complex chemical composition suggests additional functions. EW consists of minute crystals varying in size and shaped like filaments, rods, platelets, tubes, and complex dendritic structures (Barthlott et al., 2017; Jeffree, 1986). The precise mechanism of how EW reduces attachment of herbivores is not known, but four hypotheses were proposed by Gorb and Gorb (2002): (1) the aggregates of EW increase roughness of the plant surface, thus reducing the potential contact area between herbivore

foot pads and plant surface, (2) EW crystals contaminate foot pads and impair their function, (3) EW crystals draw lipophilic pad secretions away from their contact points by capillary adhesion, thus disrupting wet adhesion and lastly (4) the pad secretions (partially) dissolve EW crystals and form a thick layer of colloidal aggregate that disrupts the normal attachment process.

Orchids are among the largest families of flowering plants. Although mostly known for their spectacular floral diversity, orchids have a substantial anatomical diversity of the leaves as well (Dressler, 1981; Stern, 2014). Despite the large variation in epi- and subcuticular protective structures, orchids suffer from herbivore damage, both in the wild as well as in cultivation, which may be particularly detrimental to many endangered species in nature (Light and Macconaill, 2012) and causes huge annual capital losses to orchid nurseries worldwide (Hollingsworth and Armstrong, 2003). Common invertebrate orchid pests are slugs and snails (Hollingsworth and Sewake, 2002; Watson, 2002).

Compared to the many publications on orchid-insect herbivore biology and ecology (Light and Macconaill, 2014; Lucas-Barbosa, 2016; Subedi et al., 2011; Winkler et al., 2005), little has been published on understanding orchid-snail herbivory. Terrestrial snails and slugs adhere to and traverse many types of surfaces by using a thin layer (~10-70μm) of mucus secreted by the sole surface. To propel themselves, gastropods create a series of pulses by muscles in the foot that interact with the substrate through mucus secreted by the animal (Chan et al., 2005; Lai et al., 2010). Shirtcliffe et al. (2012) hypothesized that the amphiphilic (possessing both hydrophilic (water-loving) and lipophilic (fat-loving) properties) nature of the mucus plays an important role in adhesion of snails to many different types of surfaces. These authors based their hypothesis on the fact that they were able to reduce snail adhesion using a weak surfactant (a compound that lowers the interfacial tension between a liquid and a solid) that changed the wetting response of the surface of plant pots to the sole surface. The mucus layer helps the snail to create a stable attachment to any substrate at various inclinations (Lai et al., 2010). Adhesive locomotion on a smooth surface is less costly in terms of mucus production as compared to a rough surface (McKee et al., 2013). A recent study of Krings et al. (2019) showed that the radula is also involved in increasing mechanical interlocking with a substrate while feeding on a rough or wavy surface.

During fieldwork in tropical and temperate regions over the past twenty years, we observed that some orchid species are much more affected by herbivory than others (Gravendeel pers. comm.). We hypothesize that this phenomenon is caused by multiple factors, and that one of these factors involves leaf ornamentation acting as deterrent to herbivores. To test our hypothesis that orchid leaf ornamentation is involved in anti-herbivore defense, we investigated (i) the leaf anatomy and histology of four different orchid species, two epiphytic ones with leaves placed along the rhizome, and two terrestrial species with leaves in a basal rosette, with Scanning Electron Microscopy (SEM), Light Microscopy (LM), and Transmission Electron Microscopy (TEM); (ii) the palatability of young, juvenile and old leaves of one of the four orchid species, either left intact or with the main epicuticular properties trimmed, and (iii) adhesion of high spired-Subulinidae and low-spired Pleurodontidae snails in relation to the presence of three different epicuticular properties: trichomes, waxes and exudates.

4.2 Material and Methods

4.2.1 Snails and orchids

We observed snail species with long and short spired shells consuming orchids in the field and in cultivation (Table 4.1 and 4.2). To represent both groups, and for practical reasons, we chose to work with (sub)adults of *Subulina octona* (Subulinidae) and *Pleurodonte isabella* (Pleurodontidae). *Subulina octona* has a slender high spired shell; the crawling animal's foot surface, the sole, is elongate and narrow; *P. isabella*'s shell is much more globose and the foot of the crawling animal has a much wider sole surface (Figure 4.1). Live *Subulina octona* snails were collected from the greenhouses of plant breeder Elstgeest potplanten by placing traps, consisting of bricks with fresh cucumber slices underneath, below tables on which orchids were stowed. Live *Pleurodonte isabella* snails were purchased from a snail shop in Rotterdam (http://www.slakkenshop.nl). Live animals were placed in plastic tubes with a hole punched in the lid for fresh air access and fed with daily refreshed cucumber or lettuce ad libitum for the duration of the experiments. Both species of snails readily consumed leaves from any of the four orchid species investigated.

Table 4.1. Overview of different characteristics and epicuticular properties of four species of orchids investigated in this study.

Orchid species	Life strategy	Plant habit	Epicuticular properties of leaves								
			Leaf Position	Wax	Fine scale ridges	Exudates	Trichomes				
							Location	Length (mm)	Density (/mm2)	Diameter (mm)	Lignin in hairs
Orchis mascula	deciduous	tr.	basal	present on both sides	present	absent	absent on both sides	absent	absent	-	absent
Calanthe triplicata	evergreen	tr.	basal	absent	absent	absent	present on abaxial side	0.084±0.01	ca. 20±3 hairs	0.01± 0.001	absent
Dendrochilum pallidiflavens	evergreen	ep.	along rhizome	absent	absent	present	present on abaxial side	0.04±0.006	ca. 16±4 hairs	0.02± 0.001	absent
Trichotosia ferox	evergreen	ep.	along rhizome	absent	absent	absent	present on both sides	0.18± 0.04-1.0± 0.2	ca. 8±2-14±2 hairs	0.02± 0.004-0.03± 0.004	Present

*tr. terrestrial, ep. epiphyte

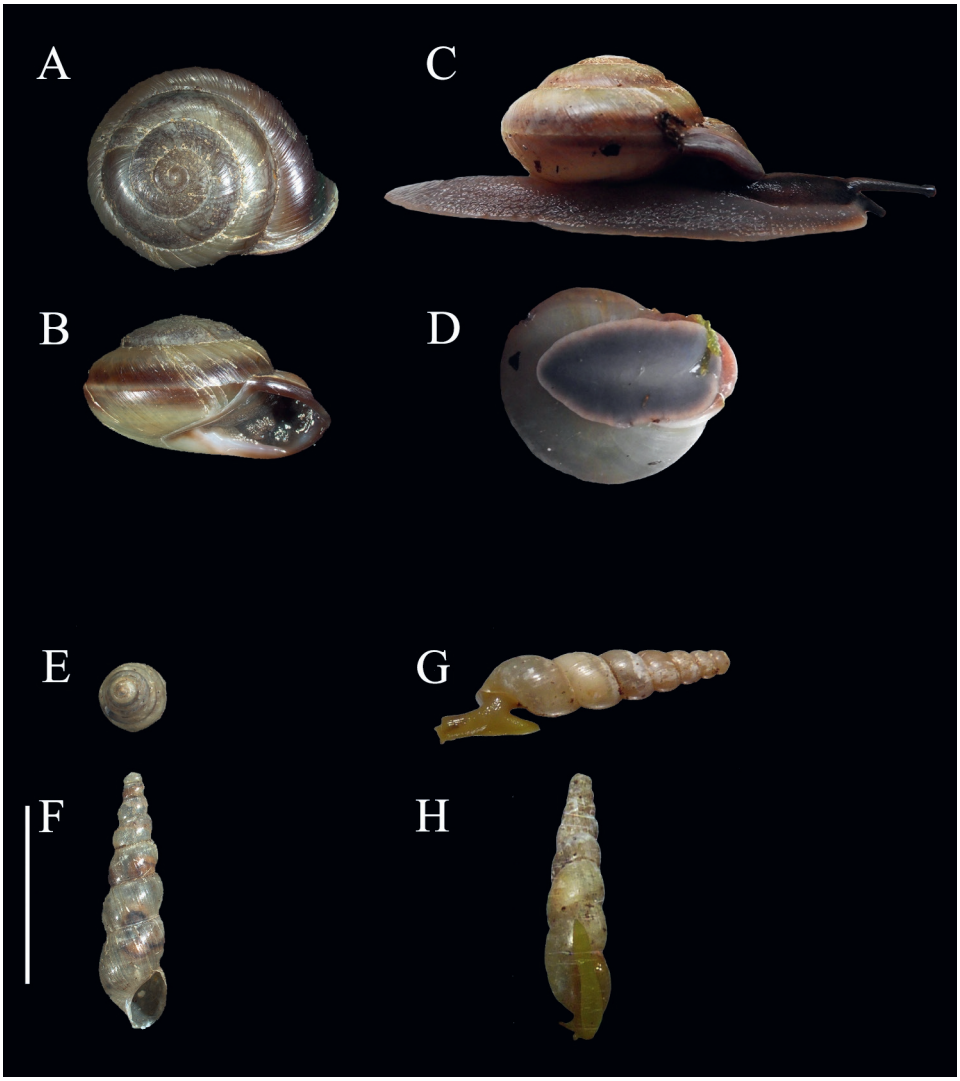


Figure 4.1. Different views of shells and live snails of the two species used in this study, *Pleurodonte isabella* (A-D) and *Subulina octona* (E-H) to illustrate the species' differences in size and shape. A, E, apical view of shells; B, F, apertural view of shells; C, G, extended (crawling) snails in lateral view; D, H, snail soles (crawling surface) attached to glass plate. Scale bar = 10 mm. Photographs by Anton J. de Winter.

We measured total mass, total surface in lateral view, and sole surface when viewed from below for both species of snails for a subset of 6-7 mature individuals per species from different size classes. Body mass was measured to the nearest 0.05 gram using a ProScale weighing scale. The lateral projected area was measured from lateral photographs, and sole surface area was measured from photographs of snails moving actively on a glass surface using ImageJ (Schneider et al., 2012). From these data, we used the correlation between body mass and lateral area to estimate the lateral area based on body mass for all specimens.

The herbivores investigated were a representative selection of snails eating from the leaves of four different locally common orchid species: the evergreen terrestrial *Calanthe triplicata* (Willemet) Ames (subfamily Epidendroideae - subtribe Collabiinae) and the epiphytic *Trichotosia ferox* Blume (subfamily Epidendroideae - subtribe Eriinae), both recorded in Manusela National Park in Seram, Indonesia in lower montane rainforest, between 1195 - 1272 m asl); the epiphytic *Dendrochilum pallidiflavens* Blume subfamily Epidendroideae - subtribe Coelogyninae) growing on Mount Salak in Java (800 – 1400 m asl); and the deciduous terrestrial *Orchis mascula* (L.) L. (subfamily Orchidoideae - subtribe Orchideae) growing in Grachterbos near Geulle, The Netherlands in mixed lowland Quercus and Carpinus coppice vegetation on a limestone slope, 61 m asl).

4.2.2 Light microscopy

Freshly harvested leaf samples were processed into microscopic slides to detect lignin, polysaccharides, carbohydrates, and calcium following protocols of Sheehan and Hrapchak (1980) and Dashek (2000). Leaf samples were first embedded in Paraffin Paraplast® Plus (Kendall Health Care Products, Japan) by rinsing the fixed samples in water and dehydrating them in a series of ethanol: xylene solutions. Then, they were stored in xylene for eight hours, infiltrated in Paraffin Paraplast® Plus (Kendall Health Care Products, Japan), and placed in an oven at 60°C for one day. Infiltrated samples were solidified and sectioned at 4–8 µm thickness with a Leica RM2265 rotary microtome (USA). Collected paraffin ribbons were laid in a 40-45ºC water bath, mounted on microscope slides, and dried on a hot plate set at 55ºC overnight. Deparaffination of samples was performed in

Table 4.2. Details of herbivorous snails and orchids that were found together, both in cultivation and in the field in The Netherlands or Indonesia.

Herbivore and orchid species	Localities in cultivation and natural habitat
Calanthe triplicata	Manusela National Park, Moluccas, Indonesia
Curvella sp. (Subulinidae)	Elstgeest potplanten, Nieuwe Wetering, The Netherlands
Subulina octona (Subulinidae)	Cibodas Botanic Gardens, Java, Indonesia
Ariophanta sp. (Ariophantidae)	Kampung Loa Loa, Moluccas, Indonesia
Orchis mascula	Grachterbos, Geule, The Netherlands
Aegopinella nitidula (Oxychilidae)	Hortus botanicus, Leiden, The Netherlands
Arion sp. (Arionidae)	
Cepaea hortensis (Helicidae)	
Cepaea nemoralis (Helicidae)	
Cochlodina laminate (Clausiliidae)	
Discus rotundatus (Discidae)	
Helix pomatia (Helicidae)	
Merdigera obscura (Enidae)	
Monachroides incarnatus (Hygromiidae)	
Trochulus hispidus (Hygromiidae)	
Trichotosia ferox	Manusela National Park, Moluccas, Indonesia
Leptopoma sp. (Cyclophoridae)	
Dendrochilum pallidiflavens	Cibodas Botanic Gardens, Java, Indonesia
Curvella sp. (Subulinidae)	

a series of xylene: ethanol solutions and the following stains were applied to the paraffin sections: an aqueous solution of Toluidine Blue O (TBO) 1% (w/v) in 1 % (w/v) sodium borate for 30 seconds to detect mucins, Etzold's staining (Basic Fuchsin 10 mg, Safranin 40 mg, Astra Blue 150 mg, Acetic acid 2 ml, and distilled water to complete 100 ml) for 3 min to detect lignin, Periodic Acid-Schiff (PAS) staining for 5 min the detection of insoluble polysaccharides and starch (Ruzin, 1999) and van Kossa (Sigma-Aldrich) for 30 min to visualizing calcium crystals. All sections were mounted in Entellan® (Merck) after dehydration and examined under a Axiolab 5 (Zeiss, Cambridge) directly after staining.

4.2.3 Scanning Electron Microscopy (SEM)

Fixed leaves were dehydrated for 20 minutes in a series of ethanol solutions (70%-96%-≥99.9%) and twice in fresh acetone ≥99.8%. Critical-point drying using ≥99.8% acetone and liquid CO2 as exchange fluids was performed in an Automated Critical Point Dryer Leica EM CPD300 (Leica Microsystems, Wetzlar, Germany). The drying protocol included a cooling step at 15°C, 50% stirrer speed with auto version, slow CO2 influx in the pressure chamber, with a delay of 120 seconds after influx of CO2 and before starting the exchange process, 18 exchange cycles (CO2: 99.8% acetone), with a fast (10 s) heating speed and medium (1 min) gas out speed. Dried samples were mounted on stubs with adhesive carbon conductive tabs and sputter-coated with 20 nm of Pt/Pd in a Quorum Q150TS (Quorum Technologies Ltd, East Sussex, United Kingdom) sputter-coater. The resulting samples were observed with a JEOL JSM-7600F Field Emission Scanning Electron Microscope (JEOL Ltd, Tokyo, Japan), at an accelerating voltage of 10 kV.

4.2.4 Transmission Electron Microscopy (TEM)

Fresh leaves were fixed in modified Karnovsky fixative (2.5% glutaraldehyde, 2% formaldehyde, in 0.1M sodium cacodylate buffer, pH 7.2) for 3 hours in a turntable and rinsed three times in 0.1M sodium cacodylate buffer (pH 7.4). Staining was performed in the dark for at least 2 hours in 2% osmium tetroxide in 0.1M sodium cacodylate buffer, and rinsing three times with 0.1M sodium cacodylate buffer (pH 7.4). Fixed samples were dehydrated in a proportion of ethanol (30%,50%,70%, 96% with 1% UAR-EMS uranyl acetate replacement,

and twice in ≥99.9% ethanol) for 15 minutes, each step in a turntable. The ≥99.9% ethanol was later replaced by propylene oxide in two steps of 15 minutes each. The sample were infiltrated in Epon (21.1% DDSA, 47.5% Embed 812, 29% NMA and 2% BDMA, all from Electron Microscopy Sciences) by submerging them in a mixture of propylene oxide and Epon (2:1, 1:1, 1:2) for 20 minutes for each step. After overnight evaporation of the remaining propylene oxide, the samples were placed in fresh Epon for 3 hours in a turntable at room temperature. Later, moderate vacuum pressure was applied for 20 minutes. The samples were embedded in fresh Epon in plastic molds and polymerized at 60°C for 48 hours. Resulting Epon blocks were trimmed in a rotary microtome with glass knives. Then, ultrathin sections of 95 nm were cut with a Leica EM UC7 ultratome (Leica Microsystems, Wetzlar, Germany), with a diamond knife and mounted on film-coated copper slot grids and post stained with uranyl acetate and lead citrate. Resulting samples were observed and photographed with a JEM-1400 PlusTEM (JEOL Ltd, Tokyo, Japan).

4.2.5 Feeding experiment

We performed feeding experiments for *S. octona* with *C. triplicata* as we had access to sufficient live animals and plants of these two species. Sterilized plastic containers were each supplied with one individual snail that had been starved for 4 days. This individual was subsequently fed with a freshly cut (2x2 cm sized) leaf piece of *C. triplicata*. Remaining leaf fragments were removed after 10 days and measured from digital photographs using ImageJ (Schneider et al., 2012). The experimental treatments were: (1) leaves either young (maximum of 1 week old), juvenile (1-3 weeks) or old (older than 3 weeks); (2) leaves with or without trimmed trichomes. Trichomes were trimmed using adhesive tape. Each treatment was replicated 8 times with different animals of comparable size and weight.

4.2.6 Attachment forces

To measure the attachment forces of the herbivorous snails investigated, we used a centrifuge technique similar to the method described by Federle et al. (2000). A part of a freshly cut leaf was clipped under two strips of acrylic (80 x 18 mm), held in place by two small magnets, which was placed on a horizontally orientated

turntable (radius r=80·mm) mounted on a rotor (Figure 4.2). We used a strobe light synchronized to the revolutions of the centrifuge through a photoelectric barrier so that a standing image of the snail on the rotating surface could be observed. The centrifuge was filmed from above (distance 50 cm) with a Nikon D5 camera. Cycle duration (in ms) was recorded with an optical tachometer, and the output was displayed on a display on the upper side of the centrifuge housing so that the speed of rotation was visible in the video image.

The snails were placed on a piece of freshly cut orchid leaf, either with the main veins perpendicular to the herbivore or in parallel and on the adaxial (=facing towards stem) or abaxial side (=facing away from stem). Individual herbivorous snails were placed on the turntable facing its centre, and the centrifuge was slowly accelerated at 0.231cycles*s-2 once it could be visually assessed that the snail had attached itself to the piece of orchid leaf. As soon as the snail flew off the leaf, the acceleration was stopped. Between every experiment, the orchid leaf fragment was refreshed. The force due to centripetal acceleration of the disk (Fc) was calculated based on the individual body mass in kilograms (Mb), the distance of the centre of the snail from the centre of rotation in meters (r) and the cycle duration in seconds (cycle) following the following formula:

$$F_c = M_b\, r \left(\frac{2\pi}{cycle}\right)^2$$

The air resistance force due to the drag (Fd) was calculated from air density (ρ), the lateral projected area (A_l) of the snail in m2 estimated based on its individual body mass, and the cycle duration in seconds (cycle) following the formula below (assuming a drag coefficient of 1):

$$F_d = 0.5\ \rho\ A_l \left[\left(\frac{2\,\pi\, r}{cycle}\right)\right]^2$$

The magnitude of the total force (Ft) parallel to the surface of the centrifuge disk needed for removal of the snail was calculated from the centrifugal force and the air drag acting perpendicular to it using the formula below:

$$F_t = \sqrt{\left({F_c}^2 + {F_d}^2\right)}$$

For each snail species, we conducted experiments with ten different animals per orchid species. For each animal, we did three consecutive measurements to not mix naive with experienced animals. We took the maximum value for analysis. The animals were allowed to recover for at least 15 min between these three measurements. Temperature and humidity were measured during every experiment to correct for their influence on air density (Federle et al., 2004).

4.2.7 Statistical analyses

To compare attachment performance across individuals that differ in mass and size, we used relative centrifugal force (RCF) rather than absolute force in our statistical analyses, as absolute force depends on the individual's mass. We did not include the forces induced by air drag or disk acceleration in the statistical analysis, as we found these to be negligible (<1% of the total force). Mean RCF was calculated from the three replicates per specimen, resulting in a single value for each snail. To identify effects of the variables that we tested (perpendicular, parallel, abaxial and adaxial position), we then performed a crossed ANOVA using the package LME4 (Bates et al., 2015) in R version 3.5.0 (R Core Team, 2018) with log-transformed mean RCF as the response variable, snail species and individual as nested independent variables, and orchid species, leaf side, leaf direction and individual as separately nested independent variables. The individual was coded as a random variable. We also tested between a more elaborate model that included the log-transformed mass of each specimen (LogMeanRCF ~ (Snail/(1 | Specimen)) *logMass + Orchid/Side/Direction). The model comparison found the more elaborate model to be only a marginally better fit than the simpler model excluding mass (p=0.0508).

To test if surface properties had a significant effect on the attachment RCF, we also tested a model containing the only variables that varied across and within orchid species: trichome length and density. Unfortunately, our dataset did not include enough differences in the presence of wax and exudates to test the effect of these two variables on attachment. The absence of trichomes was coded as very short trichomes (10-6mm), to allow log transformation. The variables

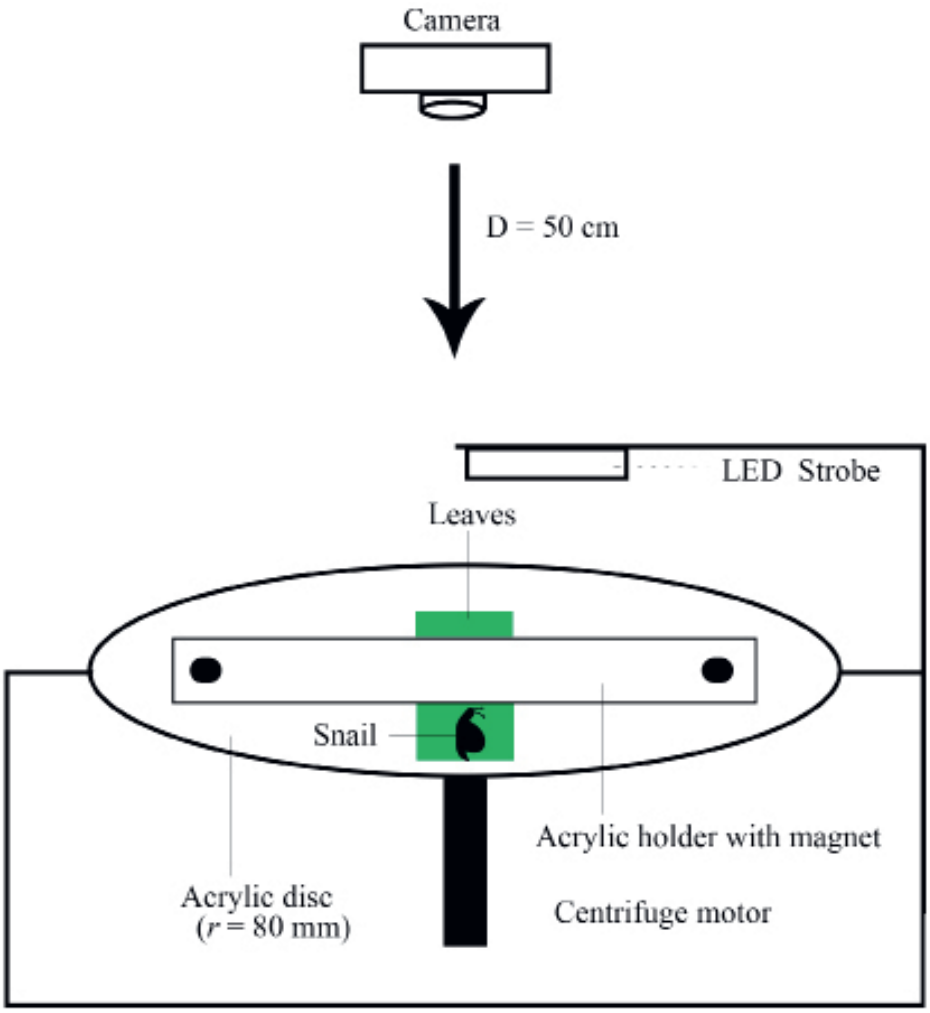


Figure 4.2. Schematic illustration of the centrifuge method used for measuring surface attachment forces of two different species of herbivorous snails. The snail was placed on a freshly cut piece of orchid leaf that was clipped to an acrylic strip on a horizontally rotating platform, which was accelerated until the snail became detached. A high-speed video camera recorded the run from above and was used to determine the maximum force of detachment. D, distance from the camera to the centre of the rotating platform; r, radius of platform.

snail species, snail specimen, and leaf side and direction were coded as nested random variables. Because we expected the effect of trichome length and density to have an interaction, we initially designed the model with that interaction. As this interaction term had no effect, it was removed. We here present the results of the following mixed model (variations of this model did not significantly alter the outcome) regarding the trichome variables: LogMeanRCF ~ (Snail / (1|Specimen)) + Orchid / (1|Side) / (1|Direction) + LogTrichomeLength + LogTrichomeDensity.

4.4 Results

4.4.1 Epicuticular properties

Detailed SEM, TEM, and LM images of the four different orchid species investigated revealed various epicuticular structures of orchid leaves surface (Figure 4.3). Both the abaxial and adaxial side of the leaves of *O. mascula* were found to be covered by an epicuticular wax layer. In contrast, *C. triplicata* has short non-glandular trichomes (ca. 0.1 mm long) in a relatively high density (ca. 20/mm2) on the abaxial side of the leaves. The leaves of *D. pallidiflavens* are covered by short glandular trichomes (ca. 0.5 mm long) on the abaxial side in a relatively low density (ca. 16/mm2) that secrete exudates. Leaves of *T. ferox* are covered by relatively large (ca. 0.2-1.6 mm long) paired trichomes on both sides. The density of these trichomes is higher on the abaxial (ca. 14/mm2) than the adaxial side (ca. 8/mm2).

The TEM images revealed a relatively thick wax layer along the cell wall of the parenchyma of the leaves of *O. mascula* on both the adaxial and abaxial side. This wax layer was thicker and more finely wrinkled on the adaxial side (ca 160-210 nm thick) as compared to the abaxial side (ca 30-40 nm thick). The non-glandular trichomes of *C. triplicata* and *T. ferox* have a thicker cell wall as compared to the glandular trichomes of *D. pallidiflavens*. We detected lipid droplets inside the glandular trichomes of *D. pallidiflavens*. The different stainings applied revealed that the paired trichomes on the leaves of *T. ferox* contain lignin. Proteins and polysaccharides were detected in the trichomes on the leaves of *C. triplicata*, *D. pallidiflavens*, and *T. ferox*. Exudates on the leaves of *D. pallidiflavens* were identified as polysaccharide substances by periodic acid staining.

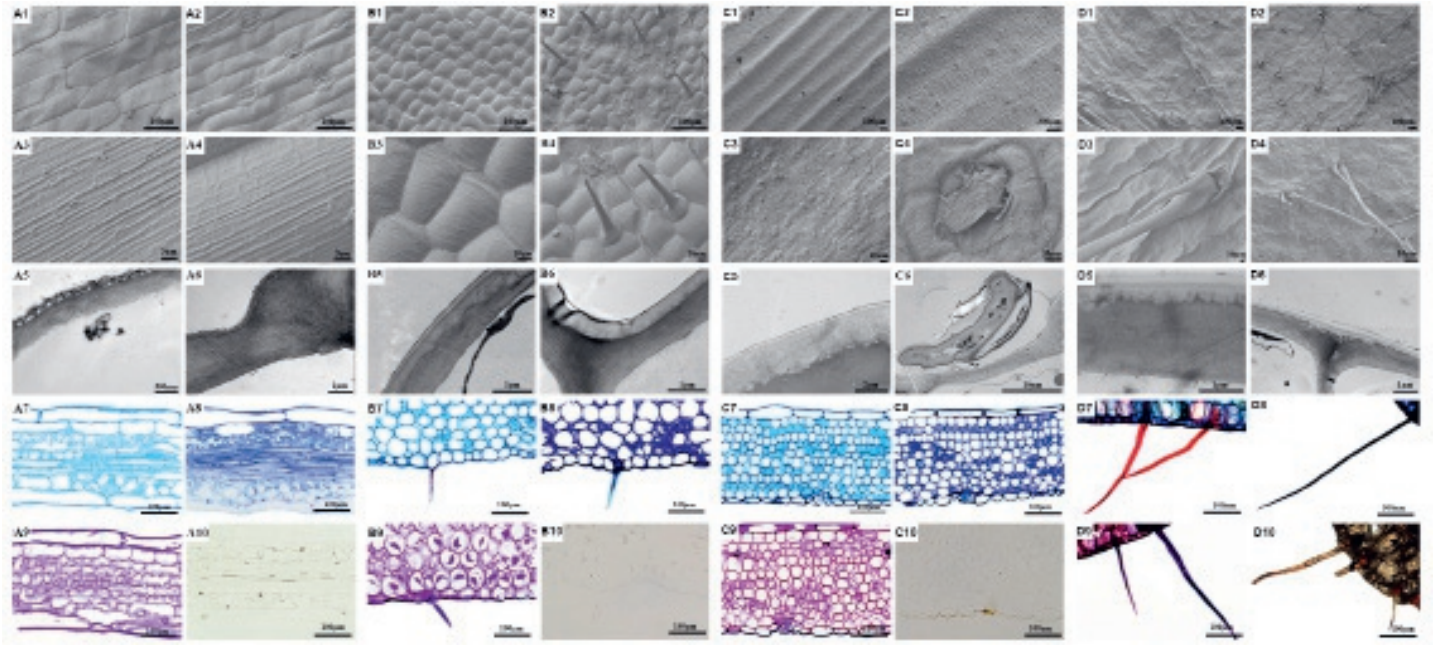


Figure 4.3. Leaf anatomy and histochemistry of orchid species used in the experiments: A1. Parenchyma cells on adaxial side of leaf of *O. mascula*. A2. Parenchyma cells on abaxial side of leaf of *O. mascula*. A3. Ornamentation of wax layer on adaxial side of leaf of *O. mascula*. A4. Ornamentation of wax layer on abaxial side of leaf of *O. mascula*. A5. Roughly waved thick wax layer on adaxial side of leaf of *O. mascula*. A6. Finely waved thinner wax layer on abaxial side of leaf of *O. mascula*. B1. Parenchyma cells on adaxial side of leaf of *C. triplicata*. B2. Parenchyma cells on abaxial side of leaf of *C. triplicata*. B3. Parenchyma cell surface on adaxial side of leaf of *C. triplicata*. B4. Trichomes on abaxial side of leaf of *C. triplicata*. B5. Section through epidermal surface on adaxial side of leaf of *C. triplicata*. B6. Section through epidermal surface on abaxial side of leaf of *C. triplicata*. C1. Ornamentation of adaxial side of leaf of *D. pallidiflavens*. C2. Ornamentation of abaxial side of leaf of *D. pallidiflavens*. C3. Parenchymal cell surface on adaxial side of leaf of *D. pallidiflavens*. C4. Trichome on abaxial side of leaf of *D. pallidiflavens*. C5. Section through epidermal surface on adaxial side of leaf of *D. pallidiflavens*. C6. Trichome basal cell on abaxial side of leaf of *D. pallidiflavens*. D1. Surface of parenchymal cells on adaxial side of leaf of *T. ferox*. D2. Surface of parenchymal cells on abaxial side of leaf of *T. ferox*. D3. Paired trichomes on leaf of *T. ferox*. D4. Paired trichomes on leaf of *T. ferox*. D5. Section through epidermal surface on adaxial side of leaf of *T. ferox*. D6. Section through epidermal surface on abaxial side of leaf of T. ferox. 7-10: Histochemistry of parenchymal cells of the epidermis of leaves of *O. mascula* (A), *C. triplicata* (B), *D. pallidiflavens* (C), *T. ferox* (D). A-D7. Staining with Etzold (lignin), A-D8. Staining with TBO (proteins), A-D9. Staining with PAS (polysaccharides), A-D10. Staining with van Kossa (calcium).

4.4.2 Feeding experiments

We found that *S. octona* preferred young leaves of *C. triplicata* over juvenile and old leaves (see Supplementary Figure 1). The mean proportion of remaining leaf fragments of untrimmed leaves was significantly larger (2.53 cm2) as compared to trimmed fragments (1.15 cm2) (Figure 4.4).

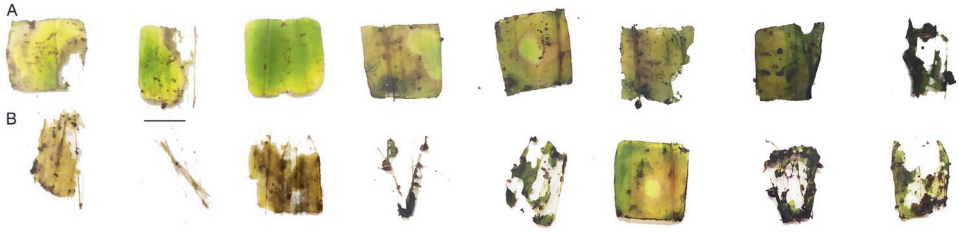


Figure 4.4. Feeding experiments result of *C. triplicata* grazed by *S. octona* after 10 days. A. untrimmed leaves; B. trimmed leaves. Scale bar: 1 cm.

4.4.3 Attachment performance

The snails that were used in the centrifuge experiments differed in size, shape, and sole surface area. We compared the weight and sole surface area for both species for 6-7 individuals per species from different size classes (Figure 4.5). The mass scaled with sole surface area with an exponent of 1.35 (95% confidence interval: 1.30-1.41) across the two species, a little less than the theoretical isometric value of 1.5. Despite this non-isometric scaling, *P. isabella* had 2.5 times more mass (0.0106 g/mm2) per unit of sole surface area than *S. octona* (0.00424 g/mm2). Both the position of the snail to the main veins on the orchid leaves, either perpendicular or in parallel ($p=0.0087$, see Table 4.3), and the side of the orchid leaf, either adaxial or abaxial ($p=0.030$), was found to influence RCF needed for snail removal. These findings are discussed in more detail below.

We ran the mixed models of mean RCF against predictor variables. Since the difference in fit between the model that included mass, and that which excluded mass was marginal, with the results being very similar between the two models, we here will mostly present the results of the simpler mixed model, excluding mass. Where

Table 4.3. Analysis of Deviance Table (Type III Wald F tests with Kenward-Roger df) with mean. Dependent variable is the log of the mean of three observations of detachment RCF per snail specimen. Formula: LogMeanRCF ~ (Snail/(1|Specimen)) + Orchid/Side/Direction. P-values that are significant at α=0.05 are shown in bold face.

Independent variables	F	Df	Df.res	Pr(>F)
(Intercept)	903	1	162	**< 2.2e-16**
Snail Species	83.9	1	159	**2.53e-16**
Orchid Species	52.2	3	144	**< 2.2e-16**
Orchid: Side	2.36	4	144	0.0561
Orchid: Side: Direction	3.09	8	144	**0.00301**

the differences between the models are of interest to understand the role of mass and snail species in attachment, we will refer to the more elaborate model, including mass.

Snail and orchid species and direction had a significant correlation (see Table 4.4). Leaf side was only marginally significant in the overall analysis. However, the orchids *T. ferox* and *D. pallidiflavens* showed significant differences in attachment RCF between leaf sides (Table 4.5). Of all the orchid species, *T. ferox* stood out as different from the other species, as RCF necessary for detachment was much lower on this species. There was a significant effect of direction on detachment RCF for *O. mascula*, with snails aligned in parallel with the axis of the leaf of this species requiring higher RCF than those oriented perpendicular to the axis of the leaf. In the more elaborate model (see Table 4.6), including snail specimen mass, snail species was non-significant (p=0.12), and mass was marginally non-significant (p=0.067), while their interaction term was also non-significant (p=0.83).

We found that trichome density and length both had a significant effect on detachment RCF, even taking variables like orchid species and leaf side into account. Higher trichome density had a negative effect on detachment RCF, while trichome length had a positive effect on detachment RCF.

We also calculated the force necessary to remove a snail from a leaf surface (Figure 4.6). The component of force due to centripetal acceleration (CF) was always much larger than that of air resistance (AD; ADmean/CFmean= 0.0043. The safety factor calculation (the snail weight divided by total force) showed that both of the snail species had poor attachment on *T. ferox* (Supplementary Figure. 2).

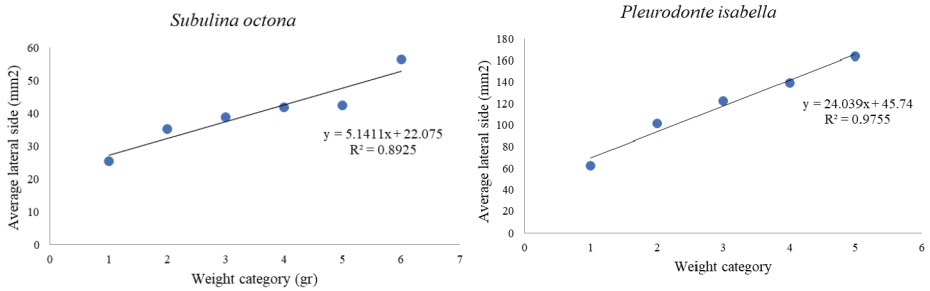


Figure 4.5. Relationship between body mass and footpad area (mm2) of *S. octona* and *P. isabella*. Weight category for *S. octona*: 1 (0.05 g), 2 (0.07 g), 3 (0.08 g), 4 (0.09 g), 5 (0.1 g); 6 (0.11 g). Weight category for *P. isabella*: 1 (2.3 g); 2 (2.4-2.6 g); 3 (2.7 g); 4 (3-3.1 g); 5 (3.2-3.3 g).

4.5 Discussion

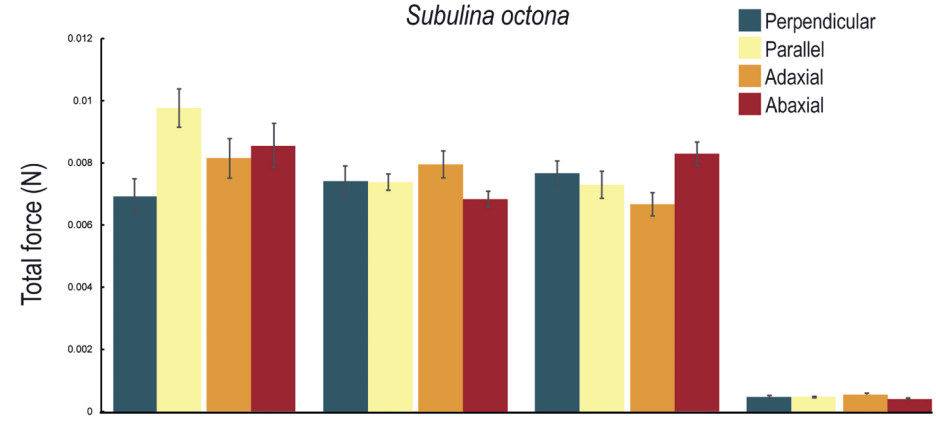
For *O. mascula*, wax layers were detected on both the adaxial and abaxial sides of the leaves. Wax layers on the epidermis of many plants species function as barrier against herbivores, to reduce water loss, and as a selective medium for spectral light penetration and reflection (Barthlott et al., 2017). The adaxial part of plant leaves, oriented parallel to the soil such as the basal leaves of *O. mascula*, is more exposed to sunlight and, therefore possibly covered with a thicker wax layer, as found in this study. The lipids detected in the wax layer have hydrophobic properties and could act as anti-adhesive for herbivorous snails (Shirtcliffe et al., 2012). The presence of epicuticular wax on the leaves of *O. mascula* might reduce the adhesion of the mucus of snails to the surface. The fact that a larger force was needed to remove the *P. isabella* snails from the abaxial as compared with the adaxial side of the leaves of *O. mascula* could be explained by our TEM observations of a slightly higher surface roughness created by fine folds of the epicuticular wax layer on the adaxial side. Such a fine pattern might significantly disrupt the attachment area below the sole surface of the larger snails. This result is in line with a study by Prüm et al. (2012) that showed that traction forces of Colorado potato beetles on plant surfaces covered by cuticular folds were reduced with 88 percent in comparison to smooth plant surfaces. According to our TEM

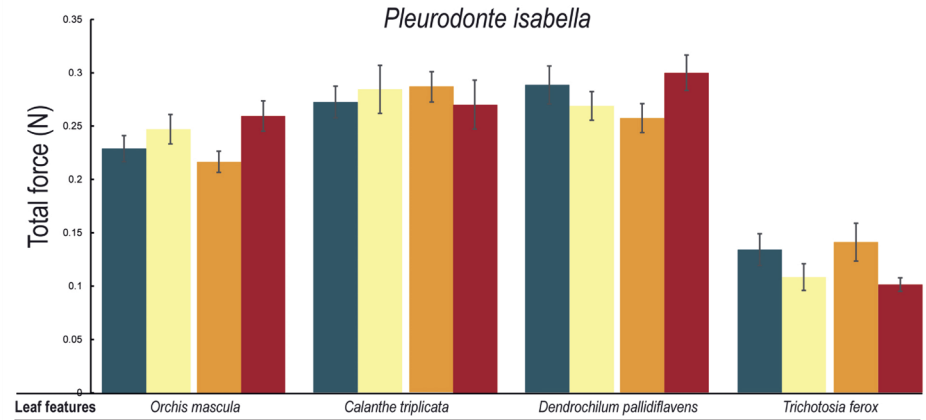
Table 4.4. Linear mixed model fit by REML. t-tests used the Satterthwaite's method ['lmerModLmerTest']. The dependent variable is the log of the mean of three observations of detachment RCF per snail specimen. P-values that are significant at α=0.05 are shown in bold.

Fixed effects	**Estimate**	**Std. Error**	**df**	**t value**	**Pr(>|t|)**
(Intercept)	0.817	0.0971	303	30.1	**< 2e-16**
Snail	0.121	0.269	303	9.16	**< 2e-16**
Dendrochilum pallidiflavens	0.0739	0.209	303	1.98	0.0485
Orchis mascula	0.0792	0.311	303	2.12	0.0345
Trichotosia ferox	-0.323	0.0375	303	-8.66	**2.88E-16**
Calanthe triplicata: Side	0.0278	0.0378	303	0.745	0.457
Dendrochilum pallidiflavens: Side	-0.0819	0.0373	303	-2.20	0.0288
Orchis mascula: Side	0.0334	0.0378	303	0.895	0.376
Trichotosia ferox: Side	0.0674	0.0371	303	1.81	0.0718
Calanthe triplicata: Abaxial: Direction	-0.0316	0.0371	303	-0.846	0.398
Dendrochilum pallidiflavens: Abaxial: Direction	0.00509	0.0372	303	0.136	0.892
Orchis mascula: Abaxial: Direction	0.0166	0.0380	303	0.446	0.656
Trichotosia ferox: Abaxial: Direction	-0.0345	0.0372	303	-0.925	0.356
Calanthe triplicata: Adaxial: Direction	0.0323	0.0371	303	0.866	0.387
Dendrochilum ferox: Adaxial: Direction	0.0366	0.0373	303	0.983	0.327
Orchis mascula: Adaxial: Direction	-0.170	0.0374	303	-4.57	**7.24E-06**
Trichotosia ferox: Adaxial: Direction	0.0231	0.0371	303	0.619	0.536

Table 4.5. Mean detachment RCF and force for each combination of factors.

Snail Species	Orchid Species	Side	Direction	RCF(G)	Force(N)
S. octona	*O. mascula*	Adaxial	Perpendicular	7.47	0.00618
S. octona	*O. mascula*	Adaxial	Parallel	15.7	0.00765
S. octona	*O. mascula*	Abaxial	Perpendicular	13.9	0.0101
S. octona	*O. mascula*	Abaxial	Parallel	11.4	0.00942
S. octona	*C. triplicata*	Adaxial	Perpendicular	9.48	0.00818
S. octona	*C. triplicata*	Adaxial	Parallel	8.54	0.00665
S. octona	*C. triplicata*	Abaxial	Perpendicular	7.95	0.00774
S. octona	*C. triplicata*	Abaxial	Parallel	8.43	0.00703
S. octona	*D. pallidiflavens*	Adaxial	Perpendicular	8.30	0.00699
S. octona	*D. pallidiflavens*	Adaxial	Parallel	7.28	0.00833
S. octona	*D. pallidiflavens*	Abaxial	Perpendicular	9.52	0.00634
S. octona	*D. pallidiflavens*	Abaxial	Parallel	9.41	0.00824
S. octona	*T. ferox*	Adaxial	Perpendicular	5.81	0.00567
S. octona	*T. ferox*	Adaxial	Parallel	5.30	0.00373
S. octona	*T. ferox*	Abaxial	Perpendicular	3.82	0.00520
S. octona	*T. ferox*	Abaxial	Parallel	4.76	0.00427
P. isabella	*O. mascula*	Adaxial	Perpendicular	6.09	0.205
P. isabella	*O. mascula*	Adaxial	Parallel	6.45	0.253
P. isabella	*O. mascula*	Abaxial	Perpendicular	6.73	0.228
P. isabella	*O. mascula*	Abaxial	Parallel	7.41	0.266
P. isabella	*C. triplicata*	Adaxial	Perpendicular	8.38	0.283
P. isabella	*C. triplicata*	Adaxial	Parallel	7.79	0.260
P. isabella	*C. triplicata*	Abaxial	Perpendicular	6.61	0.289
P. isabella	*C. triplicata*	Abaxial	Parallel	7.19	0.278
P. isabella	*D. pallidiflavens*	Adaxial	Perpendicular	8.17	0.275
P. isabella	*D. pallidiflavens*	Adaxial	Parallel	7.85	0.301
P. isabella	*D. pallidiflavens*	Abaxial	Perpendicular	9.09	0.238
P. isabella	*D. pallidiflavens*	Abaxial	Parallel	8.85	0.298
P. isabella	*T. ferox*	Adaxial	Perpendicular	3.58	0.158
P. isabella	*T. ferox*	Adaxial	Parallel	3.64	0.110
P. isabella	*T. ferox*	Abaxial	Perpendicular	3.00	0.124
P. isabella	*T. ferox*	Abaxial	Parallel	2.78	0.0926





Leaf features	*Orchis mascula*		*Calanthe triplicata*		*Dendrochilum pallidiflavens*		*Trichotosia ferox*	
	adaxial	abaxial	adaxial	abaxial	adaxial	abaxial	adaxial	abaxial
Non-glandular trichome								
Single trichome	-	-	-	+	-	-	-	-
Paired trichome	-	-	-	-	-	-	+	++
Lignified	-	-	-	-	-	-	+	+
Length	-	-	-	+	-	-	++	++
Density	-	-	-	+++	-	-	++	+
Glandular trichome								
Density	-	-	-	-	-	+	-	-
Wax layer								
Thickness	++	+	-	-	-	-	-	-

Figure 4.6. Attachment forces measured (in newton N) of *Subulina octona* and *Pleurodonte isabella* snails on four orchid leaf surfaces. Two positions were tested: perpendicular and parallel to the main veins and adaxial and abaxial side of the leaves. A: *Orchis mascula*, B: *Calanthe triplicata*, C: *Dendrochilum pallidiflavens*, D: *Trichotosia ferox*. Error bars mark the upper and lower 5%.

Table 4.6. Analysis of Deviance Table (Type III Wald F tests with Kenward-Roger df) with means. Dependent variable is the log of the mean of three observations of detachment RCF per snail specimen. Formula: LogMeanRCF ~ (Snail / (1|Specimen)) + Orchid / (1|Side) / (1|Direction) + LogTrichomeLength + LogTrichomeDensity. P-values that are significant at α=0.05 are shown in bold face.

Independent variables	F	Df	Df.res	Pr(>F)
(Intercept)	161	1	151	**< 2.2e-16**
Snail Species	75.9	1	159	**3.73e-15**
Orchid Species	41.8	3	33.6	**1.97e-11**
Trichome Length	10.3	1	152	**0.00163**
Trichome Density	10.0	1	153	**0.00187**

photographs, the amplitude of the wax folds is higher on the adaxial side of the leaves than on the abaxial side. This could explain the higher force needed to remove a snail from the abaxial side of a leaf of *O. mascula*.

During our field observations, we found many snails attached to the adaxial sides of leaves of shrubs in the forest surrounding the orchids during day time. The snails had retracted their soft parts into their shell and became active after sunset (Gravendeel pers. comm.). Terrestrial snails are known to do this to protect themselves against dehydration and predatory birds, mammals, insects and snakes (Heller and Ittiel, 1990). A slightly higher surface roughness of the epicuticular wax layer on the adaxial side of the leaves of O. mascula might have evolved in response to the above described behavior.

Several field studies showed that herbivore grazing is higher on young versus mature leaves (Coley, 1980; Williams-Linera and Baltazar, 2001; Zvereva and Kozlov, 2014) as found in our feeding experiment with *C. triplicata* leaves. This difference might be caused by the increasing concentration of chemical defense compounds built up during the lifespan of a leaf and needs further investigation. Lack of any lignin staining suggests that the trichomes on the leaves of *C. triplicata* are involved in evaporation only (Rosinski, 1992). The results of our feeding experiment, however, show that these trichomes do have a role in antiherbivore defense after all, indicating that trichomes lacking any lignin can still function as physical barriers against snails.

A higher force needed to be applied to remove both snail species from the adaxial side of the leaves of *T. ferox*. Leaves of *T. ferox* were found to be covered on both sides by trichomes containing lignin, with a slightly higher density of hairs on the abaxial than the adaxial side. The lower density of trichomes on the adaxial side could explain why the snails were able to hold on longer in the centrifuge experiments. This may be due to the fewer trichomes providing more uncovered leaf surface, and thus a larger area for the footpad of the snails to attach to than on the abaxial leaf side.

The glandular trichomes on the leaves of *D. pallidiflavens* did not affect the attachment of any of the snails. This result suggests that these trichomes on their own do not function as anti-herbivore defence, a hypothesis further supported by a study of Podroužková et al. (2015) that showed that faeces of *Succinea putris* and *Urticicola umbrosus* snails contained glandular trichomes of *Helianthus tuberosus*, indicating that snails readily consume leaves covered by glandular trichomes. The lipid droplets detected in the trichomes of *D. pallidiflavens* might, similar to the elaiosomes on the seeds of this orchid species, attract ants foraging for food, that keep snails away by their aggressive behavior, in this way protecting the orchid from herbivores in a mutualistic relationship (van Leeuwen, 1929; Van der Wall et al., 2005).

The adhesive footpad area is the main factor that affects the adhesive force of climbing animals such as herbivorous snails (Chan and Carlson, 2019; Labonte et al., 2016). The production of mucus as adhesive substance enables snails to traverse various surfaces (Chan et al., 2005; Pawlicki, 2004). *Pleurodonte isabella* did detach at a lower relative centripetal acceleration (RCF) than *Subulina octona*. This may be attributed to the scaling of footpad area to mass, which results in more mass per unit of footpad area as a snail gets larger. Perhaps to partially correct for this problem, the larger species of snails had a slightly larger footpad to mass ratio compared to what would be expected under isometric scaling. Isometric scaling describes the condition where objects of different size share the same shape, whereas the relationship between two size measures fits a power function with a particular scaling exponent (Vogel, 2013). Nonetheless, the larger species of snails (*P. isabella*) still had about 2.5 times more mass per unit foot area. If the snails would all detach at the same foot stress, we would expect the smaller species *S. octona* to detach at about 2.5 times the RCF of *P. isabella*. This

was not the case, as *S. octona* detached at 1.37 times the RCF of *P. isabella*. This difference in foot stress may be due to size-related effects of the surfaces of the orchid leaves, a stronger adhesive mucus, or shell shape (ovate versus elongate) and size-related and different sole surface effects of the snails (e.g. elongate versus more oblong shape). The scale-related factors of leaf irregularity, snail sole surface area, mucus layer thickness and physical properties of the mucus could also interact to explain the observed results. This may be an interesting avenue to explore in future research.

The differences between the models with and without mass as an independent variable seem to indicate that much of the difference in attachment RCF between the two snail species may be mediated by their difference in mass. However, when including mass in the model, the variance seemed to be divided between species and mass, leading to a marginal non-significant effect for both these variables. Even when including mass, some variance in attachment RCF may be explained by snail species. The non-isometric scaling of the snails and the potential scaling effects suggested above may contribute to this effect.

We found significant differences in detachment RCF across orchid species. The lowest values were seen on *T. ferox*, both trichome density and length are significant factors affecting detachment RCF. The safety factor results showed that both snails attached poorly on the lignified trichomes of *T. ferox*. Interestingly, increasing trichome density decreased attachment, but increasing trichome length increased attachment. Trichome density was earlier shown to have a negative effect on ovipositional behavior, feeding and larval nutrition of insect pests (Handley et al., 2005). Increasing herbivore attachment with increasing trichome length was also recorded by Voigt et al. (2007) for *Dicyphus errans* bugs on the surface of *Brassica oleracea* leaves, with a significant positive correlation between force and both trichome length and diameter. According to these authors, the trichomes provide extra grasp for the claws, thus enabling a stronger attachment of the bug. Expanding this study to more orchid species with relatively long trichomes might provide more insights in the overall effect of trichome length on snail detachment. Trichomes may reduce wet adhesion by creating large asperities on a surface. A wet adhesion mechanism is created by producing a thin fluid layer that creates capillary and viscosity forces between a pad and the surface (Hanna and Barnes, 1991). The texture of the attachment surface is an important factor for this

mechanism. A study by Crawford et al. (2016) revealed that the wet adhesion of tree frogs was strong on a smooth surface or a surface with small asperities (< 10 μm) but not on a surface with large asperities. This might be explained by insufficient production of fluid to fill the space between the large asperities and creating air bubbles that reduce the attachment to the surface. Alternatively, the large asperities may increase the drainage of fluid from the pad. Both trichomes and epicuticular wax ornamentation could thus disrupt wet adhesion of snails and other herbivores to leaf surfaces.

A waxy layer did not result in a lower detachment RCF. In *O. mascula*, the only species with a waxy outer layer, the smaller snail species *Subulina* was able to attach to an RCF of over 10g. However, *O. mascula* was the only species that showed differences in detachment RCF by direction. Our observation that a higher force and a higher RCF was needed to remove the *S. octona* snails from leaves of *O. mascula* when placed parallel to the main veins as compared with a perpendicular position might be caused by the surface roughness created by fine folds of the epicuticular wax on the leaves of this orchid species. The orientation of this fine ornamentation, as revealed by our SEM photographs is generally perpendicularly oriented to the main parallel longitudinal venation. It is yet unclear why orchid leaves evolved small-scale epicuticular wax in this orientation. The wax morphology is affected by the wax content, composition and cuticle permeability between glaucous and non-glaucous near-isogenic lines (Zhang et al., 2013). The waxes contain fatty acids, primary alcohols, esters, aldehydes, alkanes, hentriacontane-14, 16-dione (β-diketone), and OH- β-diketone that vary depending on the organs by which they are produced (Wang et al., 2017). A possible adaptive explanation might be that the main parallel veins running towards the tip of the leaf ensure quick removal of debris and small insects during heavy rainfall. The main veins, predominantly the one on the midrib, channel down raindrops towards the tip. Under the weight of accumulating raindrops, such leaves start to droop until all the water falls off the pointed apex, commonly called a ‘drip tip’. If a drip tip is cut off, rainwater starts to pool (Burd, 2007), and the leaf will become more vulnerable to infection by pathogens and less effective in photosynthesis. Small-scale roughness of orchid leaves possibly evolved in a direction perpendicular to the main veins to improve water drainage towards the main veins. In addition, such a pattern might disrupt the attachment area below the

sole surface area of small herbivorous snails. This in turn could change the area/edge ratio of the attachment or may cause stress concentrations. These hypotheses need further experimental validation.

Whether leaf surface ornamentation indeed helps plants to rid themselves of snail herbivores, depends of course on the forces that may be induced by wind or rain to a leaf. Plant appendages seem to act as damped oscillators (Peltola et al., 1993; Spatz and Bruechert, 2000) with a random input by wind or rain. The two tested snail species detached at accelerations between 2.0 g and 24.2 g (19.6-237 m/s2). Although it seems that at least the lower end of this range can be reached in plants swaying in the wind (Finnigan, 2000), measurements of g-forces on leaves exposed to natural conditions will be necessary to confirm if this is a viable mechanism for the plants included in this study to eject snails. In terrestrial species, low wind, and a small travel distance needed for the snail to regain its position on the plant may make this mechanism less viable. In epiphytic species, however, loss of footing by the snail may result in it tumbling several meters to the ground, with a lower likelihood of the snail returning to the same plant.

Our study is the first to quantify adhesion forces of herbivores on orchids. The accelerations applied were in the natural range of those induced by wind or rain to plant leaves. Our measurements provide a first estimate of total forces needed to detach herbivorous snails from orchid leaves. A high density of calcium and lignin containing hairs, and a thick wax layer were found to be effective in inducing loss of footing of the snails, possibly due to reduction of the contact area of the sole. Of the leaf epicuticular compounds detected with staining in our study, cutin, lignin, lipids, and polysaccharides such as cellulose are hydrophobic (de Candolle, 1813; Schönherr, 1982) whereas one particular group of carbohydrates, e.g., sugars, are lipophobic. Further studies on the hydrophilic and hydrophobic capacities of the epicuticular protective structures of the leaves investigated here, next to their chemistry and nanostructures as detected for several other plant species (Barthlott et al., 2017) might reveal additional details by which orchids protect themselves against snail herbivores. This might ultimately contribute to the design of a bio-coating with sufficient anti-adhesive properties. Alternatively, the attachment ability of snail pests can be decreased by the selection of orchid individuals with a relatively high amount of leaf trichomes and waxes to develop new cultivars and improve conservation of species in botanic gardens.

Supplementary material

Supplementary information is available online at Figshare, https://doi.org/10.6084/m9.figshare.13060046.v1

Bioprospecting of wild orchids

Chapter 5

Antimicrobial activity of necklace orchids is phylogenetically clustered and can be predicted with a biological response method

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Abstract. Necklace orchids (Coelogyninae, Epidendroideae) have been used in traditional medicine practices for centuries. Previous studies on a subset of unrelated orchid species utilized in these traditional practices revealed they possessed antimicrobial, anti-inflammatory, and anti-oxidant activity, providing experimental proof for their medicinal properties. To date however none of these species have been investigated ethno-botanically in a phylogenetic context. This study thus carried out comparative bioprospecting for a group of wild orchids using EBDCS (the Economic Botany Data Collection Standards) organ targeted and biological response methods. The traditional medicinal use of necklace orchids was recorded from books and journals published between 1984 and 2016. Two orchids, *Coelogyne cristata* and *Coelogyne fimbriata*, were selected, cultivated both indoors and outdoors, and the antimicrobial properties on extracts from their leaves and pseudobulbs tested against a selection of human pathogens. A molecular phylogeny of Coelogyninae based on nuclear ribosomal ITS and plastid matK DNA sequences obtained from 148 species was reconstructed with Maximum Likelihood (ML) using RAxML, Maximum Parsimony (MP) using PAUP, and Bayesian Inference using MrBayes. Bioprospecting comparison of EBDCS and biological response was carried out using customized R scripts. Ethanolic extracts obtained from leaves of *C. fimbriata* inhibited growth of *Bacillus cereus, Staphylococcus aureus,* and *Yersinia enterocolitica*, confirming the antimicrobial properties of these extracts. Leaf extracts were found to have slightly stronger antimicrobial properties for plants cultivated outdoors than indoors. These differences were not found to be statistically significant though. Three hot nodes with high potency for antimicrobial activities were detected with the EBDCS organ targeted classification method, and eight hot nodes were detected with the biological response classification method. The biological response classification method is thus a more effective tool in finding hot nodes amongst clades of species with high medicinal potential.

5.1 Introduction

For millennia, products of nature have been an important source for traditional medicine (Cragg and Newman, 2013). Even today, between 70% and 95% of the world population in developing countries continues to use traditional medicines (Robinson and Zhang, 2011). Plant-based antibiotics form the basis of these traditional medicinal systems (Newman et al., 2000). There is an increasing interest in the study of these plant-based antibiotics as a source of novel antibiotics that human pathogens may not have developed resistance against (Savoia, 2012; Cragg and Newman, 2013; Ernst et al., 2016).

To discover potential new plant species with antimicrobial properties, a time-efficient and systematic approach is needed. Bioprospecting is an approach combining phylogeny with ethnobotanical knowledge to identify potential sources of bioactive compounds. The underlying assumption is that phylogenies can predict the traditional medicinal use of natural products in a bioprospecting approach (Saslis-Lagoudakis et al., 2012; Leonti et al., 2013; Ernst et al., 2016). The hypothesis is that closely related species share similar biochemical pathways and that the same bioactive compounds are present in all descendants of a single ancestor rather than in species scattered over unrelated clades. This method has been used in different plant species (Douwes et al., 2008; Zhu et al., 2011; Saslis-Lagoudakis et al., 2012; Siqueira et al., 2012; Leonti et al., 2013) and animal groups (Smith and Wheeler, 2006). For bioprospecting, two different methods are mainly used. The first method is the Economic Botany Data Collection Standard (EBDCS) classification method. The EBDCS provides a system where cultural plant uses are described using standardized descriptors and terms, and attached to taxonomic data sets. This classification is based on the treatment of symptoms, i.e. a medicine against stomach pain (Cook, 1995). The other method is a classification based on the biological response, such as a medicine with antimicrobial effects (Ernst et al., 2016).

Pathogens cause an array of diseases in humans, and their identification is important in administering the correct treatment (Washington, 1996). It is expected that bioprospecting based on biological responses will produce different results from the organ targeted EBDCS method, as biological responses are focussed on a classification based on a single effect in the entire human body rather than

a single organ (Ernst et al., 2016). A growing number of studies report on the bioprospecting of medicinal plants, including orchids (Beena, 2011; Purkayastha, 2016). We have not yet come across any study carried out on a group of wild orchids from a phylogenetic perspective.

The orchid family is historically well well-known for its medicinal properties (Lawler, 1984; Singh and Singh, 2012). Medicinal orchids contain phytochemicals such as alkaloids, bibenzyl derivatives, flavonoids, phenanthrenes and terpenoids, which are present in leaves, roots, pseudobulbs (modified stem parts for water and nutrient storage), and flowers (Gutiérrez, 2010; Hsiao et al., 2011; Pant, 2014). Necklace orchids (Coelogyninae, Epidendroideae) comprise over 680 species, that are distributed throughout Southeast Asia (Pridgeon et al., 2005). *Bletilla*, *Coelogyne*, *Dendrochilum*, *Otochilus*, *Pholidota*, *Pleione*, and *Thunia* are examples of necklace orchid genera with documented medicinal properties (Singh and Duggal, 2009; Subedi et al., 2011; Pant and Raskoti, 2013; Teoh, 2016) (see Figure 5.1).

In this study, we (i) compiled traditional medicinal uses of necklace orchids from the literature, (ii) carried out bio-assays on six human pathogens with ethanol and hexane extracts of leaves and pseudobulbs from *C. cristata* and *C. fimbriata* plants grown both inside a glasshouse and outside to experimentally validate whether traditional growth methods impacted the orchid medicinal properties, and (iii) investigated whether an organ-targeted EBDCS or biological response-based classification was most informative for predicting the biological activity of related species.

5.2 Materials and methods

5.2.1 Medicinal uses of necklace orchids recorded in the literature

Information on the medicinal use of different species of necklace orchids was compiled from scientific journals and books throughout September 2019. We included all data from publications which stated the local names, latin names and the traditional uses for the orchid species. We excluded the publications where only the local name and genus were given. All records were compiled into a list and coded according to the Economic Botany Data Collection Standard (EBDCS) as recommended by the Biodiversity Information Standards of the Taxonomic

Databases Working Group (TDWG) (Cook, 1995). The medicinal properties of the orchid species were categorized into EBDCS level 2 characters and into biological response characters. We used the antimicrobial response character as defined in MedlinePlus with three different states: no response, possible response or unknown response. This definition assumes that a plant species should be categorized as no response when it is applied for something other than antimicrobial treatments, such as bone fracture treatments. A possible response was scored when the disease dictionary of MedlinePlus dictated this. Reducing fever was for instance scored as a possible antimicrobial effect since fever is a biological response to infection. Finally, an unknown response was given if no records of plant use were available. Medicinal properties of all *Glomera* species were categorized as unknown, as to the best of our knowledge no ethnobotanical information for this genus has been published.

5.2.2 Antimicrobial activity

5.2.2.1 Plant material

Fresh pseudobulbs and leaves of mature sterile plants of *C. cristata* and *C. fimbriata* (3-5 different individuals per species) grown in greenhouses were obtained from Orchideeën Wubben (Hollandsche Rading, The Netherlands) and Claessen Orchideeën (Nederweert-Eind, The Netherlands). The same species were subsequently grown outside where they were exposed to UV light and herbivorous snails and insects during a period of five months in the Hortus botanicus (Leiden, the Netherlands). A second batch of fresh pseudobulbs and leaves was then harvested from these species. All leaves and pseudobulbs were sterilized and freeze-dried in a VirTis Benchtop Pro Freeze Dryer at -104° C until they reached a constant weight. The dried pseudobulbs and leaves were ground into a fine powder and about 1 g of the powder was extracted with 70% ethanol and 100% hexane in a vacuum speed extractor E-916 (Buchi, Switzerland) (40°C,100 bar). The extracts were stored in a freezer (-20 °C) before further use.

5.2.2.2 Bacterial strains

The antimicrobial properties of extracts of pseudobulbs and leaves of *C. cristata*

Figure 5.1. Examples of medicinally used necklace orchids investigated in this study. A. *Pholidota imbricata*. B. *Coelogyne fimbriata*. C. *Coelogyne cristata*. D. *Coelogyne fuscescens*. The *Pholidota* species depicted under A can only be cultivated in a humid greenhouse. The *Coelogyne* species depicted under B-D can be cultivated outdoors during the summer and early fall in temperate regions to stimulate the production of secondary compounds. Photographs by Rogier van Vugt.

and *C. fimbriata* were evaluated with five bacterial strains that are common causes of human gastrointestinal tract infections and are resistant against a range of synthetic antibiotics (Mutsaers et al., 2001). Two strains of Gram positive *Staphylococcus aureus* ATCC 12600 and *Bacillus cereus* ATCC 14579 and three strains of Gram negative *Escherichia coli* ATCC 10798, *Klebsiella pneumoniae* ATCC BAA-3079, and *Yersinia enterocolitica* ATCC 9610 bacteria were used for the experiments. The bacterial strains were provided by the University of Applied

Sciences Leiden, The Netherlands. All strains were cultured on Columbia Agar with 5% Sheep Blood (COL-S (BDTM)) overnight at aerobic conditions at 37˚C (except for *Y. enterocolitica*, which was typically cultured at 30˚C), followed by storage at 4˚C for up to one week.

5.2.2.3 Antimicrobial activity of plants extracts

A disk diffusion method was used to evaluate the antimicrobial activity for each plant extract. Each bacterial strain was streaked onto a plate, grown overnight, and used to inoculate Mueller-Hinton cation-adjusted agar broth 2 (Sigma-Aldrich). The cultures were incubated overnight under aerobic conditions at 37°C (except for *Y. enterocolitica*, which was grown at 30°C) on a rotary shaker (180 rpm) until a McFarland Standard of 0.5 (107 CFU/ml) was reached. The cultures were subsequently used to make a confluent growth on COL-S agar plates. Sterile filter paper disks (10 mm diameter, Sigma-Aldrich) were loaded with the different plant extracts with a total content of 55 mg/ml. The disks were then evaporated by air at room temperature inside a laminar air flow hood for 20 min before they were placed onto the top of the inoculated plates. Sterile filter paper disks loaded with 7,5 µg of Levofloxacin (Sigma-Aldrich) were used as positive control, and sterile paper disks loaded with 5% DMSO (Sigma-Aldrich) were used as the negative control. All the samples were then incubated at 37°C (except for *Y. enterocolitica* at 30°C) for 24 h. All tests were performed in triplicate and the zones of inhibition were measured with an automatic Vernier calliper. The scale of the inhibitory effect was scored as follows: high (diameter zone ≥17 mm), intermediate (14 ≤ diameter zone < 16 mm), low (diameter zone ≤ 13 mm) (CLSI, 2011).

5.2.3 Phylogenetic reconstructions

5.2.3.1 Plant sampling and DNA extraction

Previously generated DNA sequences for necklace orchids (Gravendeel et al., 2001; Subedi et al., 2011; Sulistyo et al., 2015; Pedersen et al., 2020) were downloaded from NCBI GenBank (see Table 5.1 for more details). In addition, new DNA sequences were generated from 77 specimens of the necklace orchid genus *Glomera*. From these, 14 specimens were collected in the field in Seram,

Papua and Papua New Guinea (Indonesia). The identification of fresh material from the field was done in the Bogor Botanical Garden by Richa Kusuma Wati. Additionally, a total of 42 specimens from the living orchid collection of the Hortus botanicus in Leiden, The Netherlands were analysed. Lastly, 21 dried herbarium specimens from the Herbarium Bogoriense, Indonesia and the herbarium of Naturalis Biodiversity Center, Leiden, The Netherlands, were analysed.. Total genomic DNA was extracted from 50 mg of leaf tissue from herbarium or silica-gel dried material using the 2x CTAB (Cetyltrimethylammonium bromide) method of Doyle and Doyle (Doyle and Doyle, 1987), or with the Qiagen DNeasy Plant mini kit (Qiagen) following the manufacturer's protocol.

5.2.3.2 Amplification and Sanger sequencing

The nuclear ribosomal ITS-5.8S-ITS2 (nrITS) region of silica-gel dried leaf material was amplified using primers 17SE (5'-ACGAATTCATGGTCCGGTGAAGTGTTC-3') and 26SE (5'-TAGAATTCCCCGGTTCGCTCGCCGTTAC-3') as described by Sulistyo et al. (2015). Subsequently, a M13 universal sequencing primer was added to the 5' end of the forward (ACGAATTCATGGTCCGGTGAAGTGTTC) and reverse (TAGAATTCCCCGGTTCGCTCGCCGTTAC) primers to improve Sanger sequencing efficiency. Each PCR reaction was 25 µl and included the template DNA, CoralLoad PCR buffer (Qiagen), dNTPs, Taq DNA Polymerase (Qiagen), and both primers. A All PCR reactions were done on a C1000 Touch Thermal Cycler (Bio-Rad) instrument. The thermal cycling protocol began with a 5 min initial denaturation at 96 °C, followed by 35 amplification cycles, each with 30 sec denaturation at 96 °C, 30 sec annealing at 50 °C, and 1 min extension at 72 °C, followed by a final 7 min final extension at 72 °C.

The nrITS region of herbarium preserved leaf material was amplified using primer p3 (5'-GACTCYCGGCAATGGATATCTCG-3') and p4 (5'-CCGCTTATTGATATGCTTAAACTCRGC-3') as described by Cheng et al. (2016) and primer efgF1 (5'-CGAGTCTTTGAACGCAAGTTGCG-3') and efgR1 (5'-GGCCAACGAGACGATAACCC-3') that were newly designed. Each PCR reaction consisted of 25 µl, containing the template DNA, 5x Phire PCR buffer (ThermoScientific), BSA, dNTPs, Phire Hot Start II DNA Polymerase

(ThermoScientific), and both primers. The thermal cycling protocol began with a 1 min initial denaturation at 98 °C, followed by 40 amplification cycles, each with 10 sec denaturation at 98 °C, 10 sec annealing at 50 °C, and 20 sec extension at 72 °C, followed by a a 1 min final extension at 72 °C.

The matK region of silica dried silica-gel dried leaf material was amplified using two primer sets: 731F (5'-TCTGGAGTCTTTCTTGAGCGA-3') and 2R (5'-AACTAGTCGGAGTAG-3'), and 19F (5'-CGTTCTGACCATATTGCACTATG-3') and 881R (5'-TMTTCATCAGAATAAGAGT-3') as described by Sulistyo et al. (2015). The PCR reaction setup was the same as that for the nrITS with fresh plant material, but with additional BSA. The thermal cycling protocol began with a 5 min initial denaturation at 94 °C, followed by 35 amplification cycles, each with a 1 min denaturation at 94 °C, 30 sec annealing at 50 °C, and 1 min extension at 72 °C, followed by a 7 min final extension at 72 °C. Sanger sequencing of the amplification products were performed at Baseclear (http://www.baseclear.com/), using an ABI-3730XL DNA Sequencer (Applied Biosystems). All sequences were deposited in NCBI GenBank. Accession numbers of all sequences can be found in Table 5.1.

Table 5.1. Details of DNA sequences generated in earlier studies that were used for the phylogenetic analyses in combination with the newly generated data of *Glomera*.

Arundina graminifolia (D.Don) Hochr.;sine loco, MWC 395 (K), AF461461, AF302692. *Bletilla ochracea* Schltr.; [accession 1] sine loco, SXDF, KF698627, -. [accession 2], China, L.Li 06 (IBSC), -, KR857335. *Bletilla foliosa* (King & Pantl.) Tang & F.T. Wang; China, BSMZ03, KP866836, -. *Bletilla striata* (Thunb.) Rchb.f.; [accession 1] sine loco, MWC556, AF461466, -. [accession 2] sine loco, KFBG316, KY966713. *Chelonistele amplissima* (Ames & C. Schweinf.) Carr; Brunei, sine coll./cult. Hort. Bot. Leiden 26834 (L), AF302730, AF302695. *Chelonistele sulphurea* (Blume) Pfitzer; sine loco, sine coll./cult. Hort. Bot. Leiden 21528 (L), AF302729, AF302694. *Coelogyne asperata* Lindl.; Papua New Guinea, sine coll./cult. Hort. Bot. Leiden 22279 (L), AF281128, AY003881. *Coelogyne barbata* Lindl. ex Griff.; India, sine coll./cult. Hort. Bot. Leiden 990040 (L), AF302755, AF302720. *Coelogyne beccarii* Rchb.f.; Papua New Guinea, sine

coll./cult. Hort. Bot. Leiden 32230 (L), AF302751, AF302716. *Coelogyne bicamerata* J.J.Sm.; Sulawesi, sine coll./cult. Hort. Bot. Leiden 931067 (L), AF302756, AF302721. *Coelogyne bilamellata* Lindl.; Philippines, sine coll./cult. Hort. Bot. Leiden 25164(L), AF302747, AF302712. *Coelogyne chloroptera* Rchb.f.; Philippines, sine coll./cult. Hort. Bot. Leiden 23511 (L), AF302760, AF302725. *Coelogyne corymbosa* Lindl.; sine loco, HQ130495, HQ130488. *Coelogyne cristata* Lindl.; sine loco, sine coll./cult. Hort. Bot. Leiden 2214 (L), AF302742, AF302707. *Coelogyne cuprea* H.Wendl. & Kraenzl.; Brunei, sine coll./cult. Hort. Bot. Leiden 914768(L), AF302748, AF302713. *Coelogyne pulverula* Teijsm. & Binn.; sine loco, AF281126, AY003879. *Coelogyne eberhardtii* Gagnep.; Vietnam, sine coll./cult. Hort. Bot. Leiden 970803 (L), AF302754, AF302719. *Coelogyne fimbriata* Lindl.; sine loco, sine coll./cult. Hort. Bot. Leiden 30759 (L), AF302745, AF302710. *Coelogyne flaccida* Lindl.; sine loco, sine coll./cult. Hort. Bot. Leiden 940707 (L), AF302743, AF302708. *Coelogyne flexuosa* Rolfe; sine loco, sine coll./cult. Hort. Bot. Leiden 19937 (L), AF302746, AF302711. *Coelogyne foestermannii* Rchb.f.; Sarawak, sine coll./cult. Hort. Bot. Leiden 970591 (L), AF281123, AY003876. *Coelogyne fuscescens* Lindl.; sine loco, SBB0612, JN114450, -. *Coelogyne harana* J.J.Sm.; Kalimantan, sine coll./cult. Hort. Bot. Leiden 970290 (L), AF302749, AF302714. *Coelogyne kelamensis* J.J.Sm.; Kalimantan, sine coll./cult. Hort. Bot. Leiden 930568 (L), AF302750, AF302715. *Coelogyne macdonaldii* F.Muell. & Kraenzl.; Vanuatu, sine coll./cult. Hort. Bot. Leiden 25836 (L), AF302752, AF302717. *Coelogyne miniata* (Blume) Lindl.; Java, sine coll./cult. Hort. Bot. Leiden 990287 (L), AF302761, AF302726. *Coelogyne multiflora* Schltr.; Sulawesi, sine coll./cult. Hort. Bot. Leiden 21747 (L), AF302758, AF302723. *Coelogyne nitida* (Wall. Ex D.Don); [accession 1] sine loco, HQ130496, -. [accession 2] sine loco, SBB-0640, - JN004373. *Coelogyne ovalis* Lindl.; [accession 1] India, DS0034, MK169302, -. [accession 2] sine loco, KFBG423, -, KY966800. *Coelogyne pandurata* Lindl.; sine loco, sine coll./cult. Hort. Bot. Leiden 21532 (L), AF281130, AY003883. *Coelogyne plicatissima* Ames & C. Schweinf.; Sarawak, sine coll./cult. Hort. Bot. Leiden 980409 (L), AF281125, AY003878. *Coelogyne prolifera* Lindl.; [accession 1] sine loco, SGLD-MO12, KF866230, -. [accession 2] India, NRCO/Gen/Cel/15/1, -, KR905391. *Coelogyne punctulata* Lindl.; sine loco, HQ130499, HQ130492. *Coelogyne rhabdobulbon* Schltr.; Sabah, sine coll./cult. Hort. Bot.

Leiden 26597 (L), AF281127, AY003880. *Coelogyne sanderiana* Rchb.f.; sine loco, sine coll./cult. Hort. Bot. Leiden 30765 (L), AF281124, AY003877. *Coelogyne stricta* (D.Don) Schltr.; sine loco, sine coll./cult. Hort. Bot. Leiden 30695 (L), AF302757, AF302722. *Coelogyne trinervis* Lindl.; sine loco, sine coll./cult. Hort. Bot. Leiden 26940 (L), AF302744, AF302709. *Coelogyne veitchii* Rolfe; Papua New Guinea, sine coll./cult. Hort. Bot. Leiden 22277 (L), AF302759, AF302724. *Coelogyne velutina* de Vogel; Malay Peninsula, sine coll./cult. Hort. Bot. Leiden 25835 (L), AF302753, AF302718. *Coelogyne brachyptera* Rchb.f.; sine loco, AF81122, AY003875. *Dendrochilum alatum* Ames; Malaysia, Sabah, Mt. Kinabalu, Barkman 137 (SNP), MG788045, -. *Dendrochilum alpinum* Carr; Malaysia, Sabah, Mt. Kinabalu, Barkman 142 (SNP), MG788047, MG788102. *Dendrochilum apoense* T.Hashim; sine loco, sine coll./cult. Hort. Bot. Hafn. P2012.5170 (C), MG788060, MG788147. *Dendrochilum arachnites* Rchb.f.; sine loco, sine coll./cult. Hort. Bot. Hafn. P2012.5211 (C), MG788076, MG788118. *Dendrochilum auriculare* Ames; sine loco, sine coll./cult. Hort. Bot. Hafn. P2012.5172 (C), MG788073, MG788137. *Dendrochilum banksii* Cootes; sine loco, sine coll./cult. Hort. Bot. Hafn. P2012.5173 (C), MG788064, MG788132. *Dendrochilum celebesense* H.A.Pedersen & Gravend.; Indonesia, Sulawesi, Gravendeel & Mudiana 697 (L), AY534,-. *Dendrochilum citrinum* H.A.Pedersen; Indonesia, Sulawesi, sine coll./cult. Hort. Bot. Leiden 22672 (L barcode L.1508201, spirit specimen), MG788031, MG788098. *Dendrochilum cobbianum* Rchb.f.; sine loco, sine coll./cult. Hort. Bot. Hafn. P2012.5174 (C), MG788024, MG788099. *Dendrochilum coccineum* H.A.Pedersen & Gravend.; sine loco, sine coll./cult. Hort. Bot. Hafn. P2012.5175 (C), MG788061, MG788086. *Dendrochilum convallariaeforme* Schauer; sine loco, sine coll./cult. Hort. Bot. Hafn. P2012.5177 (C), MG788057, MG788124. *Dendrochilum cootesii* H.A.Pedersen; sine loco, sine coll./cult. Hort. Bot. Hafn. SO-0226 (C), -, MG788142. *Dendrochilum cornutum* Blume; sine loco, sine coll./cult. Hort. Bot. Hafn. P2012.5178 (C), MG788036; MG788116. *Dendrochilum corrugatum* (Ridl.) J.J.Sm.; Malaysia, Sabah, Mt. Kinabalu, Barkman 11 (SNP), MG788046, MG788119. *Dendrochilum cruciforme* J.J.Wood; Malaysia, Sabah, Mt. Kinabalu, Barkman194 (SNP), -, MG788108. *Dendrochilum cupulatum* J.J.Wood.; Malaysia, Sabah, Crocker Range, Barkman 228 (SNP), MG788059, MG788105. *Dendrochilum curranii* Ames; sine loco, sine coll./cult. Hort. Bot. Hafn.

P2012.5179 (C), MG788081, MG788128. *Dendrochilum dewildeorum* J.J.Wood & J.B.Comber; Barkman 324 (SNP), AF76721/AF76759,-. *Dendrochilum dewindtianum* W.W.Sm.; Malaysia, Sabah, Mt. Kinabalu, Barkman 1 (SNP), MG788044, MG788109. *Dendrochilum diabloviride* Cootes & R.Boos; sine loco, sine coll./cult. Hort. Bot. Hafn. P2012.5209 (C); MG788062, MG788126. *Dendrochilum edentulum* Blume; Indonesia, Jawa Barat, sine loco, sine coll./cult. Hort. Bot. Leiden 18634 (L), MG788037, MG788117. *Dendrochilum erectilabium* H.A.Pedersen; Indonesia, Sulawesi, sine loco, sine coll./cult. Hort. Bot. Hafn. SO-0147 (C), MG788032, MG788097. *Dendrochilum exasperatum* Ames; Malaysia, Sabah, Mt. Kinabalu, Barkman 212 (SNP), MG788041, MG788113. *Dendrochilum filiforme* Lindl.; sine loco, sine coll./cult. Hort. Bot. Hafn. P2012.5180 (C), MG788027, MG788110. *Dendrochilum gibbsiae* C3200 Rolfe [accession 1]; Malaysia, Sabah, sine loco, sine coll./cult. Hort. Bot. Leiden 23447 (L), MG788039, MG788112; [accession 2]; Malaysia, Sarawak, sine loco, sine coll./cult. Hort. Bot. Leiden 1823 (L), MG788040, MG788114. *Dendrochilum glumaceum* Lindl.; sine loco, sine coll./cult. Hort. Bot. Hafn. P2012.5240 (C), MG788023, MG788111. *Dendrochilum gracile* (Hook.f.) J.J.Sm.; sine loco, sine coll./cult. Hort. Bot. Leiden 20010038 (L), MG788109, -. *Dendrochilum graciliscapum* (Ames) Pfitzer; Barkman 329 (SNP), AF076719/AF076757, -. *Dendrochilum graminifolium* (Ames) Pfitzer; Philippines, sine loco, sine coll./cult. Hort. Leiden 24616 (L), MG788070, MG788145. *Dendrochilum grandiflorum* (Ridl.) J.J.Sm.; Barkman 10 (SNP), AF76702/AF76740, -. *Dendrochilum hampelii* Sulistyo & Gravend.; sine loco, sine coll./cult. Hort. Bot. Leiden 20130654 (L), KT334203, KT334210. *Dendrochilum haslamii* Ames; Malaysia, Sabah, Mt. Kinabalu, Barkman 17 (SNP), MG788043, -. *Dendrochilum havilandii* Pfitzer; Malaysia, Sarawak, sine loco, sine coll./cult. Hort. Leiden 21185 (L), MG788034, MG788095. *Dendrochilum javierianum* Magrath, Bulmer & I.Shafer; sine loco, sine coll./cult. Hort. Bot. Hafn. P2012.5183 (C), MG788063, MG788130. *Dendrochilum joclemensii* Ames; Malaysia, Sabah, Mt. Kinabalu, Barkman 261 (SNP), MG788042, MG788115. *Dendrochilum kamborangense* Ames; Malaysia, Sabah, Mt. Kinabalu, Barkman 64 (SNP), MG788049, -. *Dendrochilum karoense* J.J.Wood; Barkman 326 (SNP), AF076726/AF076764, -. *Dendrochilum kingie* (Hook.f.) J.J.Sm.; Malaysia, Sabah, Wood C14843 (SNP), MG788033, MG788094. *Dendrochilum lacteum* Carr; Malaysia, Sabah, Mt. Kinabalu, Barkman 235 (SNP),

MG788053, -. *Dendrochilum lancilabium* Ames; Malaysia, Sabah, Mt. Kinabalu, Barkman 229 (SNP), MG788054, MG788106. *Dendrochilum latifolium* Lindl.; sine loco, sine coll./cult. Hort. Bot. Hafn. P2012.5242 (C), MG788021, MG788087. *Dendrochilum linearifolium* Hook.f.; Malaysia, Pahang, sine loco, sine coll./cult. Hort. Leiden 18633 (L), MG788035, MG788093. *Dendrochilum longibulbum* Ames; sine loco, sine coll./cult. Hort. Bot. Hafn. P2012.5186 (C), MG788074, MG788141. *Dendrochilum longifolium* Rchb.f.; Papua New Guinea, Southern Highlands, sine loco, sine coll./cult. Hort. Bot. Hafn. SO-0127 (C), MG788029, MG788090. *Dendrochilum latifolium* var. macranthum (Schltr.) H.A. Pedersen; sine loco, sine coll./cult. Hort. Bot. Hafn. P2012.5185 (C), MG788022, MG788088. *Dendrochilum magnum* Rchb.f.; sine loco, sine coll./cult. Hort. Bot. Hafn. P1991.5412 (C), MG788020, MG788089. *Dendrochilum microchilum* (Schltr.) Ames; sine loco, sine coll./cult. Hort. Bot. Hafn. P2012.5187 (C), MG788082, MG788129. *Dendrochilum muluense* J.J.Wood; Malaysia, Sabah, Mt. Alab, Barkman 349 (SNP), MG788051, MG788103. *Dendrochilum odoratum* (Ridl.) J.J.Sm.; sine loco, sine coll./cult. Hort. Bot. Hafn. P2012.5188 (C), MG788058, MG788091. *Dendrochilum ovatum* J.J.Sm.; Barkman 325 (SNP), AF076727/ AF076765, -. *Dendrochilum pallidiflavens* Blume; Indonesia, Jawa Barat, sine loco, sine coll./cult. Hort. Bot. Hafn. SO-0099 (C), MG788084, MG788148. *Dendrochilum pangasinanense* Ames; sine loco, sine coll./cult. Hort. Bot. Hafn. P2012.5189 (C), MG788025, MG788101. *Dendrochilum parvulum* (Ames) Pfitzer; sine loco, sine coll./cult. Hort. Bot. Hafn. P2012.5190 (C), MG788083, MG788138. *Dendrochilum pseudoscriptum* T.J.Barkman & J.J.Wood; Barkman 16 (SNP), AF315840/AF315842, -. *Dendrochilum pseudowenzelii* H.A.Pedersen; sine loco, sine coll./cult. Hort. Bot. Hafn. P2012.5192 (C), MG788067, MG788131. *Dendrochilum pterogyne* Carr; Malaysia, Sabah, Mt. Kinabalu, Barkman 2 (SNP), -, MG788107. *Dendrochilum pulcherrimum* (Ames) L.O.Williams; sine loco, sine coll./cult. Hort. Bot. Hafn. P2012.5193 (C), MG788078, MG788134. *Dendrochilum saccolabium* Kraenzl.; sine loco, sine coll./cult. Hort. Bot. Hafn. SO-0158 (C), MG788068, MG788139. *Dendrochilum schaiblei* H.A. Pedersen; Indonesia, Sulawesi, sine loco, sine coll./cult. Hort. Bot. Hafn. SO-0111 (C), MG788030, MG788096. *Dendrochilum scriptum* Carr; Malaysia, Sabah, Mt. Kinabalu, Barkman 9 (SNP), MG788048, MG788104. *Dendrochilum selebicum* (J.J.Sm.) H.A.Pedersen & Graveend.; Indonesia, Sulawesi, De Vogel/ cult. Hort.

Bot. Leiden 20446 (L barcode L.1487561, spirit specimen), AF281120, MG7880085. D*endrochilum septemnervium* H.A.Pedersen; sine loco, sine coll./ cult. Hort. Bot. Hafn. P2012.5195 (C), MG788055, MG788122. *Dendrochilum serratoi* (Ames) Cootes; sine loco, sine coll./cult. Hort. Bot. Hafn. P2012.5194 (C), MG788080, MG788127. *Dendrochilum simile* Blume; sine loco, sine coll./ cult. Hort. Bot. Hafn. P2012.5196 (C), MG788038, MG788092. *Dendrochilum smithianum* (Ames) Pfitzer; Philippines, Luzon, sine loco, sine coll./cult. Hort. Bot. Leiden 19431 (L), MG788079, MG788135. *Dendrochilum stachyodes* (Ridl.) J.J.Sm.; Malaysia, Sabah, Mt. Kinabalu, Barkman 8 (SNP), MG788050, MG788121. *Dendrochilum stenophyllum* L.O. Williams; sine loco, sine coll./cult. Hort. Bot. Hafn. P2012.5198 (C), MG788069, MG788146. *Dendrochilum tenellum* (Nees & Meyen) Ames; sine loco, sine coll./cult. Hort. Bot. Hafn. P2012.5199 (C), MG788071, MG788143. *Dendrochilum tenompokense* Carr; Barkman 262 (SNP), AF076715/AF076753, -. *Dendrochilum tortile* H.A.Pedersen; sine loco, sine coll./cult. Hort. Bot. Hafn. P2012.5200 (C), MG788056, MG788123. *Dendrochilum transversum* Carr; Barkman 95 (SNP), AF076694/AF076732, -. *Dendrochilum trusmadiense* J.J.Wood; Malaysia, Sabah, Mt. Trus Madi, Barkman 154 (SNP), MG788052, -. *Dendrochilum uncatum* Rchb.f.; no voucher (plant deceased), MG788026, MG788100. *Dendrochilum warrenii* H.A.Pedersen & Gravend.; sine loco, sine coll./cult. Hort. Bot. Hafn. P2012.5217 (C), MG788028, MG788125. *Dendrochilum wenzelii* Ames; [accession 1, red-flowered]: sine loco, sine coll./cult. Hort. Bot. Hafn. P2012.5204 (C), MG788066, MG788133; [accession 2, yellow-flowered]: Philippines, sine loco, sine coll./cult. Hort. Bot. Hafn. SO-0125 (C), MG788065, MG788136. *Dendrochilum williamsii* (Ames) Pfitzer; sine loco, sine coll./cult. Hort. Bot. Hafn. P2012.5205 (C), MG788072, MG788144. D*endrochilum woodianum* Ames; sine loco, sine coll./cult. Hort. Bot. Hafn. P2012.5206 (C), MG788075, MG788140. *Dendrochilum yuccifolium* L.O.Williams; sine loco, sine coll./cult. Hort. Bot. Hafn. P2012.5207 (C), MG788077, MG788149. *Entomophobia kinabaluensis* (Ames) de Vogel; Malaysia, Sarawak, sine coll./cult. Hort. Bot. Leiden L.970404, AF302732, AF302697. *Geesinkorchis phaiostele* (Ridl.) de Vogel; Borneo, sine coll./cult. Hort. Bot. Leiden L.30700, AF302733, AF302698. *Glomera acutiflora* 20030686 (Schltr.) J.J.Sm.; Papua New Guinea, Mt. Bapaoto, sine coll./cult. Hort. Bot. Leiden 20030686, L.4173439 (L), MN255133, MN255271. *Glomera amboinensis*

RK246 (Ridl.) J.J.Sm.; Indonesia, Moluccas, sine coll./cult. Herbarium Bogoriense RK246, MN255137, MN255272. *Glomera compressa* 20032455 J.J.Sm.; Papua New Guinea, Sepik-Waghi Divide, sine coll./cult. Hort. Bot. Leiden 20032455, L0301943/L4163270, MN255219, MN255273. *Glomera hamadryas* 20030203 (Schltr.) J.J.Sm.; Papua New Guinea, Mt. Alio, sine coll./cult. Hort. Bot. Leiden 20030203, MN255220, MN255274. *Glomera inconspicua* 20080771 J.J.Sm.; Papua New Guinea, sine coll./cult. Hort. Bot. Leiden 20080771, MN255153, -. *Glomera papuana* 20050958 Rolfe; Papua New Guinea, sine coll./cult. Hort. Bot. Leiden 20050958, MN255161, MN255276. *Glomera pungens* 20030329 (Schltr.) J.J.Sm.; Papua New Guinea, Mt.Silisi, sine coll./cult. Hort. Bot. Leiden 20030329, MN255164, MN255277. *Glomera secunda* RK260 J.J.Sm.; Indonesia, Moluccas, sine coll. Herbarium Bogoriense RK260, MN255173, MN255280. *Glomera uniflora* 20031761 J.J.Sm.; Papua New Guinea, sine coll./cult. Hort. Bot. Leiden 20031761, MN255214, MN255269. *Nabaluia angustifolia* de Vogel; Malaysia, Sabah, sine coll./cult. Hort. Bot. Leiden L.26217, AF302734, AF302699. *Neogyna gardneriana* (Lindl.) Rchb.f.; sine loco, sine coll./cult. Hort.Bot. Leiden L.970729, AF302735, AF302700. *Otochilus albus* Lindl.; sine loco, -, HQ130493. *Otochilus fuscus* Lindl.; sine loco, KFBG761, -, KY966912. *Otochilus lancilabius* Lindl.; sine loco, HQ130500, HQ130494. *Panisea tricallosa* Rolfe; China, sine coll./cult. Hort. Bot. Leiden L.970828, AF302736, AF302701. *Pholidota articulata* Lindl.; sine loco, KFBG383, KY966648, KY966933. *Pholidota cantonensis* Rolfe; sine loco, KFBG658A, KY966649, KY966934. *Pholidota carnea* (Blume) Lindl.; Indonesia, Sumatera, sine coll./cult. Hort. Bot. Leiden L25469, AF302737, AF302702. *Pholidota chinensis* Lindl.; sine loco, KFBG799, KY966650, KY966935. *Pholidota imbricata* Hook.; sine loco, sine coll./cult. Hort. Bot. Leiden L.21540, AF302738, AF302703. *Pholidota pallida* Lindl.; sine loco, sine coll./cult. Smithsonian Gardens US Gostel263, MH766906, MH748943. *Pleione bulbocodioides* (Franch.) Rolfe; sine loco, sine coll./cult. Hort. Bot. Leiden L.990010, AF302739, AF302704. *Pleione formosana* Hayata; Indonesia, Sulawesi, sine coll./cult. Hort. Bot. Leiden L.931067, AF302740, AF302705. *Pleione hookeriana* (Lindl.) Rollisson; China, Xing'an, MF775379, MF775392. *Pleione humilis* (Sm.) D.Don; Nepal, van den Berg C409 (K), AF461495, AF503666. *Pleione maculata* (Lindl.) Lindl. & Paxton; sine loco, van den Berg C370 (K), AF461493, AF503741. *Pleione praecox* (Sm.) D.Don; sine loco, van

den Berg C368 (K), AF461491, AF503742. *Thunia alba* (Lindl.) Rchb.f.; Chase 589 (K), AY008466, AY121731.

5.2.3.3 Sequence editing and phylogenetic analysis

Sanger sequences were assembled and edited in Geneious ® R8 (Biomatters Ltd., Auckland, New Zealand) (Kearse et al., 2012). The ends of all data sets were trimmed to avoid character misinterpretation. Ambiguous bases were replaced with "N" in the data matrix. DNA sequences were aligned using the MAFFT platform (Multiple Alignment Fast Fourier Transform) (Katoh and Standley, 2013) as implemented in Geneious ® R8 with subsequent manual adjustment. Missing data were replaced with "?".

A phylogenetic analysis was carried out using Bayesian Inference (BI) with *Arundina graminifolia* as an outgroup based on earlier studies (Gravendeel et al., 2001; Pedersen et al., 2020) that showed this genus to be most closely related to the necklace orchids. The chosen nucleotide substitution model GTR+G was calculated using the Akaike Information Criterion (AIC) in jModelTest2 v.2.1.6 (Darriba et al., 2015). The analyses were run in the CIPRES Science Gateway v.3.1. (Miller et al., 2010). We performed Bayesian interference analyses with Mr.Bayes v.3.2.6 on XSEDE (Huelsenbeck et al., 2004) with the following parameters for the alignment dataset: number of runs (nruns=2), number of chains to run (nchains=4), number of generations (ngen=5 x 107), temperature parameter (temp=2) and sampling frequency of 2000 yielding 25000 trees per run. The log files from MrBayes were inspected in Tracer v.1.6 to check for convergence of independent runs (i.e. with estimated sample size (ESS)>200). Maximum Likelihood analyses were performed with RAxML-HPC2 on XSEDE (8.2.10) (Stamakis et al., 2008) choosing the GTRGAMMA model for bootstrapping and 1,000 bootstrap iterations. Parsimony analyses were performed with PAUPRat: Parsimony ratchet searches using PAUP* (Nixon, 1999; Sikes and Lewis, 2001; Swofford, 2002) with 1000 ratchet repetitions, seed value = 0, 20% percent of characters to perturb (pct = 20), original weights 1 for all characters (wtmode = uniform) and a tree bisection-reconnection branch swapping algorithm (swap = TBR). The 50% majority rule consensus for MP was obtained with PAUP

v4.0a152. and observed in FigTree v.1.3.1. The statistical support of the clades was evaluated with the values of posterior probability (PP) for BI reconstruction, bootstrap for ML (MLB) and parsimony bootstrap for MP (MPB). The support values (PP) were added to the branches on the Bayesian 50% majority-rule consensus tree with additional support values shown for ML and MP when the same topology was retrieved.

5.2.4 Bioprospecting analysis

A randomly selected subset of 1.000 trees within the 95% highest posterior density (HPD) interval was used for further analyses using the packages caper, ape, plyr (Paradis et al., 2004; Kembel et al., 2010; Wickham, 2011; Orme et al., 2013) and scripts in the R programming language (R Core Team, 2018) under RStudio (Gandrud, 2015). The R bioprospecting script of Ernst et al. (2016) was used to assess evolutionary patterns of medicinal properties of the necklace orchids analysed. The strength of the phylogenetic signal of the EBDCS category and the antimicrobial biological response category were investigated using D statistics (Fritz and Purvis, 2010), that was calculated with the phylo.d function implemented in the R package caper (Orme et al., 2013). A boxplot of the D values for each category of the two classification methods investigated was made using ggplot. If 95% of the 1.000 trees had a median value of D>1, the medicinal properties were considered as randomly distributed; for D<1, the phylogenetic signal was considered as strong (Ernst et al., 2016). D>0 indicates that the medicinal properties of the orchids possess a significantly different distribution from the standard Brownian model, implying that they are clustered within the phylogeny. D<0 indicates that the categories are extremely clustered. The prevalence of each category was measured by Ntotal species included in the category / Ntotal number of species. For a prevalence <0,020 the category was considered as too biased, and omitted from further analyses.

We also tested the phylogenetic diversity (PD) of the EBDCS category and the antimicrobial biological response category with the function pd in the R package picante v.1.6-2 (Kembel et al., 2010). The percentage of the possible response category of the antimicrobial biological response was compared with the Infections/Infestations category of the EBDCS classification method. A higher PD

percentage means that species in this category are more scattered throughout the phylogeny. As a consequence, more potential species with medicinal properties are present because the PD-values are based on the total branch length spanned by the species (Ernst et al., 2016). A consensus BI tree with 10% burnin was used to visualize the distribution of the two categories over the necklace orchid species investigated. Using the nodesigl command in R with the system PHYLOCOM v4.2 (Webb et al., 2008), so-called 'hot nodes' were calculated to visualize potential medicinal species.

5.3 Results

5.3.1 Medicinal uses of necklace orchids recorded in the literature

For 28 necklace orchid species, traditional medicinal uses were compiled to encompass 19 organ-targeted categories and a single biological response (i.e., antimicrobial) category with three different character states (see Tables 5.2, 5.3, 5.4 for an overview of all data obtained from the literature). The prevalence of the categories Mental Disorders, Nervous System Disorders and Sensory System Disorders in the EBDCS classification method showed the lowest value of 0,006 because only one species was used in these categories.

5.3.2 Bioassays

None of the 100% hexane leaf and pseudobulb extracts and 70% of the ethanol pseudobulb extracts showed any antimicrobial effect in the bio-assays conducted. On the contrary, the 70% ethanol leaf extracts inhibited the growth of several of the human pathogens investigated (Table 5.5). Extracts from freshly harvested leaves of *C. cristata* and *C. fimbriata* inhibited growth of *Y. enterocolitica*, *B. cereus* and *S. aureus* and confirmed the traditional medicine uses recorded in the literature (Pyakurel and Gurung, 2008; Subedi, 2002; Subedi et al., 2013).

The highest effect was recorded for the 70% EtOH leaf extracts of *C. fimbriata* against *Y. enterocollitica* (19.6 + 4.2 mm). Intermediate effects were recorded for leaf extracts of the same *Coelogyne* species against *B. cereus* (14.3 + 1.4 mm) and *S. aureus* (13.6 + 1.2 mm). Leaf extracts were found to have slightly stronger (but not significant) antimicrobial properties for plants cultivated outdoors than indoors (Table 5.6).

Table 5.2. Information on traditional medicinal use of necklace orchids (Coelogyninae) compiled from the literature.

Species	Use		
	Symptoms	Plant organ(s)	References
Bletilla formosana (Hayata) Schltr.	Strengthen the lungs, stop bleeding and reduce swellings. Used for treatment of tuberculous cough, bronchiectasis, bleeding peptic ulcers, nose-bleed and treat cracks on the heel.	Stems	Teoh (2016)
Bletilla ochracea Schltr.	See *B. striata*	Tubers/ pseudobulbs	Teoh (2016)
Bletilla foliosa (King & Pantl.) Tang & F.T.Wang	See *B. striata*	Tubers/ pseudobulbs	Teoh (2016)
Bletilla striata (Thunb.) Rchb.f.	Benefit the lungs (effect on pulmonary diseases), liver and stomach meridians. Effects of the medicine are haemostatic, reduce swelling and promotes regeneration of muscles and other tissues. Also used to treat sores, pustules and dry, chapped and burned skin.	Tubers/ pseudobulbs	Teoh (2016)
Coelogyne barbata Lindl. Ex Griff.	The whole plant is valued for its ability to counter 'heat', relieve thirst, stop coughs and lessen pain. It is used to treat sore throat, pain at hernias, swelling of the scrotum, chappy extremities, traumatic injuries and 'lung-heat'	Entire plant	Teoh (2016)

Coelogyne corymbosa Lindl.	Paste applied to the forehead to relieve headaches, fresh juice applied to burns and wounds as an analgesic. It treats fractures and is used as haemostatic and to relieve pain. Reduces heat and taken for coughs, flu, and bronchitis.	Pseudobulbs/ entire plant	Pant and Raskoti. (2013); Subedi et al. (2001, 2013); Teoh (2016); Vaidya et al. (2000); Yonzone et al. (2012)
Coelogyne cristata Lindl.	Are given for constipation as well as diarrhoea and dysentery. It is also used as an aphrodisiac. Freshly collected paste or juice consumed to relieve headaches, fever and for indigestion. Pulp applied to burnt skin. Juice also applied to wounds and skin boils. Gum is used for sores. Used for cooling & soothing.	Pseudobulbs	Pant and Raskoti (2013); Subedi et al. (2001, 2013); Teoh (2016); Vaidya et al. (2000)
Coelogyne fimbriata Lindl.	Powder used in tonic preparation and used to reduce heat.	Pseudobulbs	Subedi et al. (2011, 2013); Teoh (2016)
Coelogyne flaccida Lindl.	Paste applied externally or consumed to relieve frontal headaches, fever, and boils. Juice is taken for indigestion. The whole plant is also used to clear heat, counter dryness, promote the production of body fluids, clear phlegm and stop coughs.	Pseudobulbs/ entire plant	Pant and Raskoti (2013); Subedi et al. (2011, 2013); Teoh (2016)
Coelogyne fuscescens Lindl.	Paste applied externally or consumed to relieve headaches, fever, and stomach/abdominal ache. Treat burns and otitis media. Has sometimes an aphrodisiac function.	Pseudobulbs	Pant and Raskoti. (2013); Subedi et al. (2011, 2013); Teoh (2016); Vaidya et al. (2000); Yonzone et al. (2012)

Coelogyne nitida (Wall. Ex D. Don) Lindl.	Juice consumed against headaches and fever and recommended for stomach ache. Paste applied externally on burns.	Pseudobulbs	Pant and Raskoti (2013); Subedi et al. (2011, 2013); Teoh (2016)
Coelogyne ovalis Lindl.	Used as a tonic, aphrodisiac and to treat coughs, urine infections and eye disorders	Not specified	Pant and Raskoti (2013); Teoh (2016); Yonzone et al. (2012, 2013)
Coelogyne prolifera Lindl.	Paste consumed against headaches and fever. Paste applied externally on burns, boils and to relieve backache.	Pseudobulbs	Pant and Raskoti (2013); Subedi et al. (2011, 2013); Teoh (2016)
Coelogyne punctulata Lindl.	Used to treat wounds, burns, dry coughs. Relieves pain and helps to heal the wounds.	Pseudobulbs	Teoh (2016); Yonzone et al. (2012, 2013)
Coelogyne stricta (D.Don) Schltr.	Paste applied externally against headaches and fever. Healing of fractured bones.	Pseudobulbs	Pant and Raskoti (2013); Subedi et al. (2011, 2013); Teoh (2016); Vaidya et al. (2000); Yonzone et al. (2013)
Coelogyne trinervis Lindl.	Used to treat fractures and sprains	Tuber	Teoh (2016)
Otochilus lancilabius Seidenf.	Paste applied to fractured and dislocated bones.	Entire plant	Subedi et al. (2011, 2013)
Pholidota articulata Lindl.	Paste applied on fractured bones and consumed as a tonic. Root powder is used to treat cancer. Juice berries are used to treat ulcers, skin eruptions, traumatic injuries, and sores. Removes gas and reduce swelling. Also used to treat coughs caused by body heat, headache, dizziness, irregular menses, and uterine prolapse.	Entire plant	Pant and Raskoti (2013); Subedi et al. (2011, 2013); Teoh (2016); Vaidya et al. (2000)

Pholidota cantonensis Rolfe	Used to treat high fever, eczema, and haemorrhoids.	Entire plant	Teoh (2016)
Pholidota chinensis Lindl.	Used for cooling, moistens the lungs, promotes salivation. Used to treat tuberculosis-associated haemoptysis, acute or chronic bronchitis, dry cough, pharynchitis, tonsillitis, toothache, peptic ulcer, gastroenteritis, dizziness, headache, post-concussion syndrome, neurasthenia, osteomyelitis and trauma	Entire plant	Teoh (2016); Yonzone et al. (2012); Wang J. et al. (2006)
Pholidota imbricata Hook.	Paste consumed to relieve fever and powder as a tonic. Juice is applied to relieve navel pain, abdominal pain, rheumatic pain, and headache. Applied to boils and to treat fractures.	Pseudobulbs	Pant and Raskoti (2013); Subedi et al. (2011,2013); Teoh (2016); Vaidya et al. (2000); Yonzone et al. (2012, 2013)
Pholidota pallida Lindl.	Paste used to relieve fever, powder to induce sleep and to cure abdominal pain, juice used for navel pain and rheumatic pain and sore throat.	Rhizome, pseudobulbs	Pant and Raskoti (2013); Subedi et al. (2011, 2013); Teoh (2016); Vaidya et al. (2000); Yonzone et al. (2012,2013)
Pleione bulbocodioides (Franch.) Rolfe	Treatment for wet sores, sore throat, rabies, tuberculosis, asthma, boils, and carbuncles. Clears phlegm. It reduces inflammation and fever. It removes extravasated blood swellings. It is also used as a detoxifier.	Entire plant	Teoh (2016)
Pleione hookeriana (Lindl.) Rollisson	Are used to remove heat, toxins, abscesses and lymphatic tuberculosis.	Pseudobulbs	Teoh, (2016)

Pleione humilis (Sm.) D.Don	Paste applied on cuts and wounds. Powder used as a tonic.	Pseudobulbs	Pant and Raskoti (2013); Subedi et al. (2011,2013); Teoh (2016)
Pleione maculata (Lindl.) Lindl. & Paxton	Used for liver and stomach ailments.	Pseudobulbs	Pant and Raskoti (2013); Subedi et al. (2013); Teoh (2016); Vaidya et al. (2000); Yonzone et al. (2012,2013)
Pleione praecox (Sm.) D.Don	Dried powder consumed (with milk) as tonic and energizer. Paste externally applied on cuts and wounds.	Pseudobulbs	Pant and Raskoti (2013); Subedi et al. (2011,2013); Teoh. (2016)
Thunia alba (Lindl.) Rchb.f.	Paste used on fractured and dislocated bones. Benefit the lungs, clear phlegm and stop cough, remove bruises and improve blood flow	Entire plant	Pant and Raskoti (2013); Subedi et al. (2011,2013); Teoh (2016)

5.3.3 Bioprospecting of necklace orchids

The majority consensus reconstructed BI tree, which is based on combined nrITS and plastid matK sequences for 148 species of necklace orchid species, is depicted in Figure 5.2. The consensus tree of ML and MP shows relatively high support for (>70%) and was congruent with the topology of the majority consensus BI tree. The Infections/Infestation category of the organ targeted EBDCS (Figure 5.2A) classification method and the biological (i.e. antimicrobial response) method (Figure 5.2B) were plotted on the BI tree.

The boxplots of the D-statistics for the organ-based EBDCS classification method and the antimicrobial biological response classification method are shown in Figures 5.3 and 5.4. For the EBDCS classification method, 7 of the 19 categories showed a D>1, indicating that a minority of these categories were randomly distributed. A total of 12 of the 19 categories were (extremely) clustered. For the antimicrobial response method, all the categories were found to be (extremely) clustered.

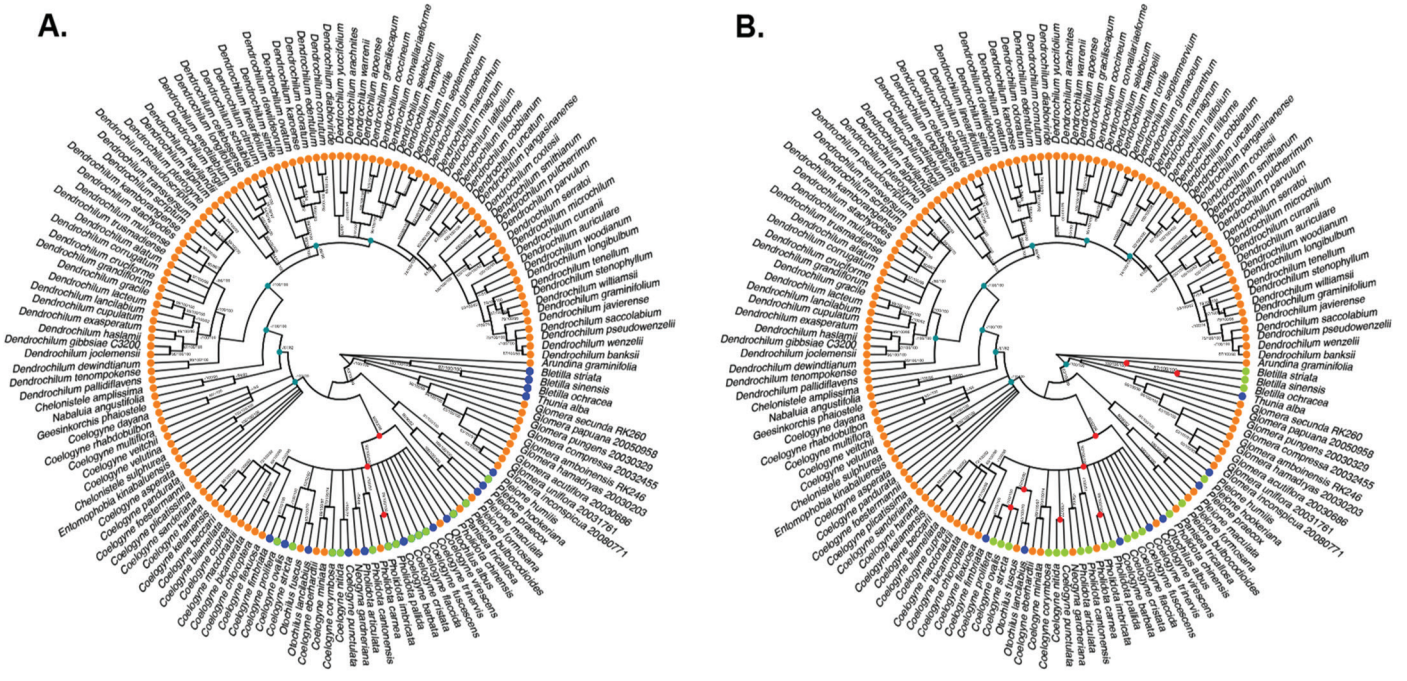

Figure 5.2. Majority consensus Bayesian Inference tree reconstructed on combined nrITS and plastid matK sequences of species of necklace orchids (Coelogyninae). A. Plotting of the Infections/Infestation category of the organ targeted EBDCS classification method on this BI tree. B. Plotting of the Antimicrobial biological response classification method. Explanation of colour codes: species with no antimicrobial use (blue), species with unknown antimicrobial use (orange), species with possible antimicrobial use (light green), ancestral hot nodes of clades with high potency of species with antimicrobial properties (red). Hot nodes were identified with the nodesigl command in the PHYLOCOM package. Plotted branch values for MLBS, MPB, and PP are given for each well-supported clade.

Table 5.3. Prevalence of various categories of medicinal use of necklace orchids for the organ targeted EBDCS classification method.

EBDCS classification method	NTotal species included in the category	Prevalence
Abnormalities	5	0.033
Circulatory System Disorders	2	0.013
Digestive System Disorders	14	0.094
Genitourinary System Disorders	5	0.033
III-defined Symptoms	2	0.013
Infections/Infestations	13	0.087
Inflammations	3	0.020
Injuries	16	0.108
Mental Disorders	1	0.006
Metabolic System Disorders	9	0.060
Muscular-Skeletal System Disorders	13	0.087
Nervous System Disorders	1	0.006
Nutritional Disorders	7	0.047
Pain	12	0.081
Poisonings	2	0.013
Respiratory System Disorders	13	0.087
Sensory System Disorders	1	0.006
Skin/Subcutaneous Cellular Tissue Disorder	13	0.087
Unknown	120	0.810
NTotal (Total number of species)	148	

Table 5.4. Prevalence of various categories of medicinal use of necklace orchids for the antimicrobial response classification method.

Antimicrobial response classification method	NTotal number of species included in the category	Prevalence
No documented response	111	0,75
Possible response	19	0,123
Unknown process	122	0,824
NTotal (Total number of species)	148	

Table 5.5. Antimicrobial activity of extracts of *Coelogyne cristata* and *C. fimbriata* as recorded in the bioassays carried out in this study of 5 plants per species grown in greenhouses. All experiments were carried out in triplicate. Absence of growth inhibition is indicated with -.

Extracts	Zone of Inhibition(mm)				
	B.cereus	*E.coli*	*K. pneumoniae*	*S. aureus*	*Y. enterocollitica*
Positive control (7.5 µg/ml levofloxacin)	22.87±1.0	13.12±0.2	22±2.2	14.6±0.6	38±1.5
70% EtOH Pseudobulbs *C. cristata*	-	-	-	-	-
70% EtOH Leaves *C. cristata*	-	-	-	-	-
Hexane Pseudobulbs *C. cristata*	-	-	-	-	-
Hexane Leaves *C. cristata*	-	-	-	-	-
70% EtOH Pseudobulbs *C. fimbriata*	-	-	-	-	-
70% EtOH Leaves *C. fimbriata*	15.55±0.6	13.88±0.7	18.55±0.6	13.3±1.0	21.7±2.0
Hexane Pseudobulbs *C. fimbriata*	-	-	-	-	-
Hexane Leaves *C. fimbriata*	-	-	-	-	-

Table 5.6. Antimicrobial activity of extracts of *Coelogyne cristata* and *C. fimbriata* as recorded in the bioassays carried out in this study of 5 plants per species grown outside for five months in the Hortus botanicus Leiden, The Netherlands. All experiments were carried out in triplicate. Absence of inhibition zone is indicated with -.

Extracts	Zone of Inhibition(mm)				
	B. cereus	E.coli	K.pneumoniae	S.aureus	Y. enterocollitica
Positive control (7.5 µg/ml levofloxacin)	22.51±0.8	18.77±0.4	26±1.2	20.15±0.2	39±2.0
70% EtOH Pseudobulbs *C. cristata*	-	-	-	-	-
70% EtOH Leaves *C. cristata*	-	-	-	-	-
70% EtOH Pseudobulbs *C. fimbriata*	-	-	-	-	-
70% EtOH Leaves *C. fimbriata*	16.44±0.8	14.55±1.4	17.22±1.0	22.55±1.4	20.44±1.1

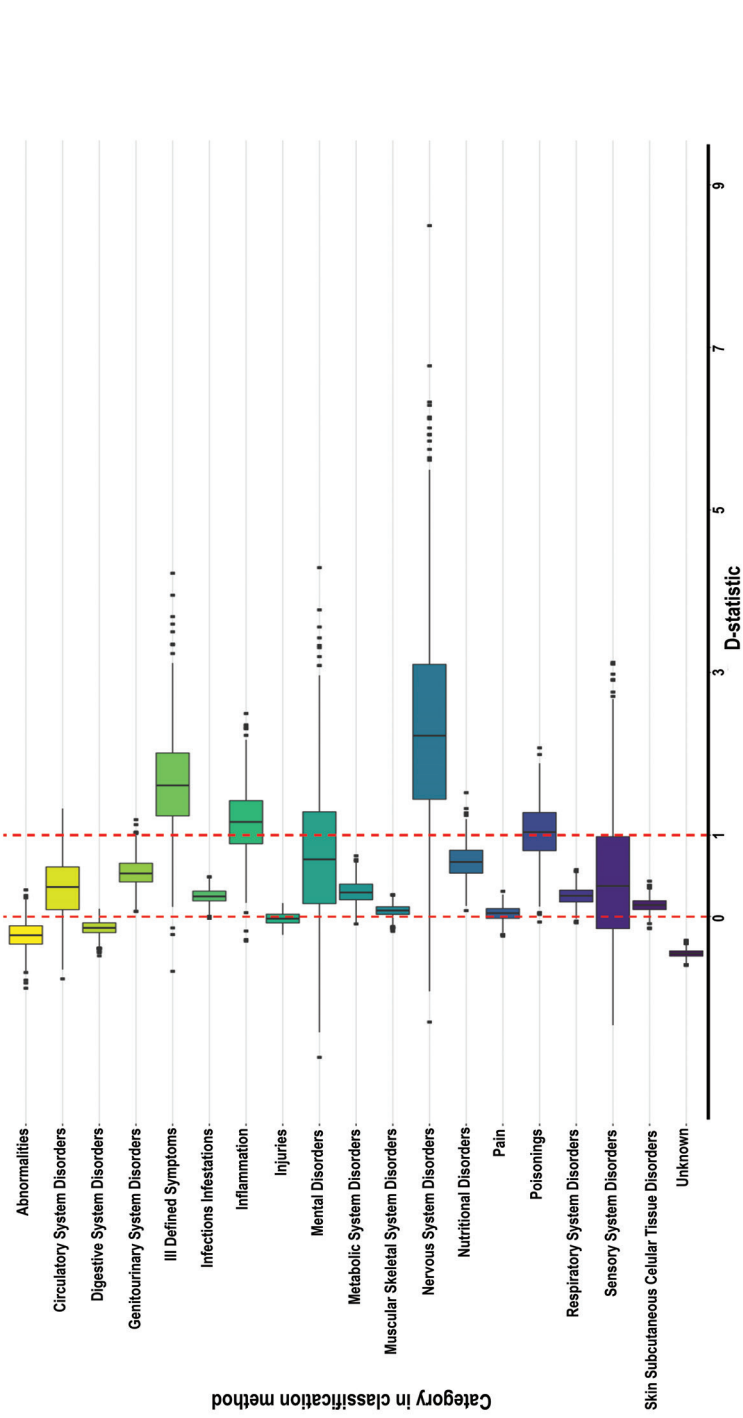

Figure 5.3. Boxplot of the 19 categories of the organ targeted EBDCS classification method (indicated with different colours) over which the data on medicinally used necklace orchid species that were analysed phylogenetically can be divided. The red lines indicate the D reference values 0 (on the left) and 1 (on the right). The box boundaries indicate the first and third quartile (Q1 and Q3), the line indicates the median, and the whiskers extend to either the extreme values or 1.5 times the interquartile range (Q3-Q1).

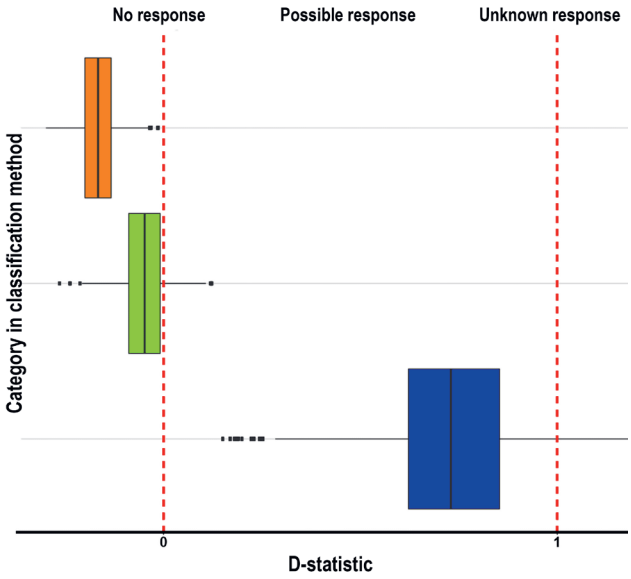

Figure 5.4. Boxplot of the Antimicrobial biological response classification method of the three characters states over which the data on medicinally used necklace orchid species, that were analysed phylogenetically, could be divided. Colour codes: no response (orange), possible response (green), unknown response (blue). The red lines indicate the D reference values 0 (on the left) and 1 (on the right). The box boundaries indicate the first and third quartile (Q1 and Q3), the line indicates the median, and the whiskers extend to either the extreme values or 1.5 times the interquartile range (Q3-Q1).

The median of the phylogenetic diversity (PD) was calculated to compare the phylogenetic distribution of medicinal species from the Unknown, Possible and No Antimicrobial Response categories with the 19 categories of the organ based EBDSC classification method. In Figure 5.5, these medians are depicted. The Possible Antimicrobial Response category of the biological classification method had a median of 18.83%, whereas the Infections/Infestations category of the organ based EBDSC classification method had a median of 13.32%.

To narrow down potential new species with antimicrobial activities, the Possible Antimicrobial Response state of the biological response classification method was compared with the Infections/Infestations category of the EBDCS classification method using the PHYLOCOM platform. Figure 5.2 depicts the recovered hot nodes. Figure 5.2A shows the three hot nodes detected for

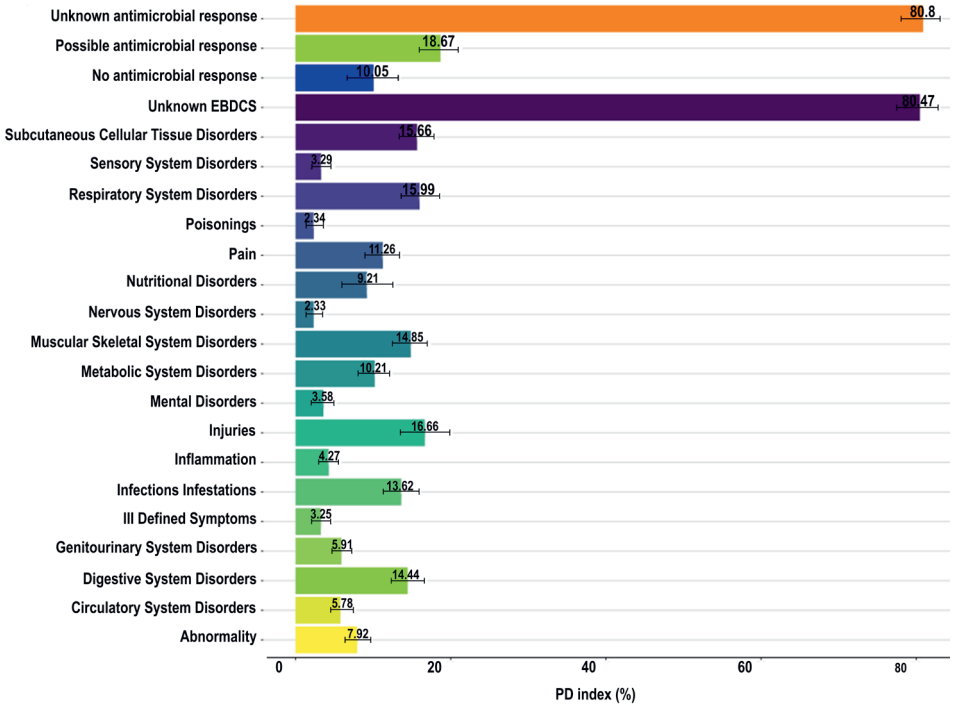

Figure 5.5. Median and standard errors of the Phylogenetic Diversity (PD) indices (in %) of the biological (i.e. antimicrobial) response and organ targeted EBDCS classification methods (indicated with different colours).

the category within the EBDCS classification method with high potency for antimicrobial activities. Figure 5.2B shows the eight hot nodes detected for the biological response classification method.

5.4 Discussion

When compiling data on medicinal use of necklace orchids from the scientific literature, we noted that information for specific species was not always provided. This was for instance the case for the genus *Dendrochilum*. We thus urge ethnobotanists to make vouchers so that more detailed information for a particular genus can be obtained to link medicinal uses to the species level.

Detailed information on plant organs used for medicinal purposes was not always provided either. We therefore urge ethnobotanists to ask more detailed questions about specific organs used when interviewing traditional plant healers working with orchids. When information about plant organs was mentioned, this was sometimes contradictory among different publications. Our bio-assays showed that antimicrobial effects for extracts of leaves from *C. fimbriata* were much higher than those for pseudobulbs, which is not fully in agreement with previous publications, where it was found that pseudobulbs were the main source of secondary metabolites (Tóth, 2018). Our results are however supported by the publication of Buyun et al. (2016), who found that leaf extracts from *Coelogyne ovalis* produced larger inhibition zones than pseudobulb extracts. The ethanol extract of leaves of *Bulbophyllum neilgherrense* showed the same result (Priya and Krishnaveni, 2005). A possible explanation might be that the metabolites present in the pseudobulbs are more diluted as the relative amount of water is usually higher in these organs than in the leaves. The exact method used to obtain plant extracts was also not always provided. Extracts dissolved in 70% ethanol had a higher antimicrobial effect in our bio-assays than extracts dissolved in hexane. This difference might be explained by the fact that hexane is a non-polar solvent that mostly extracts large fatty acid methyl esters with hydrocarbons and terpenes, whereas known antimicrobial substances isolated from necklace orchids are mostly phenanthrenes (Majumder et al., 2001; Kovács et al., 2008; Yang et al., 2012; Pant, 2014; Qian et al., 2015), which dissolve more readily in polar solvents such as ethanol.

Whether medicinal orchids were collected in the wild or from gardens or greenhouses was not mentioned in any of the publications that we screened. Our bio-assays show that antimicrobial effects of extracts of plants grown outside were higher (but not significantly so) than those of plants cultivated in greenhouses. A possible explanation for the difference in antimicrobial activity may be that plants naturally produce secondary metabolites that have a role in the defence against abiotic and biotic stresses (Dangle and Jones, 2001; Kim et al., 2009; Ramakrishna and Ravishankar, 2011).

Recent studies by Isah (2019) also show that both stress and defense responses are involved in secondary metabolite production in plants. The insignificant differences found in our experiments when comparing indoor versus

outdoor cultivation methods might be a result of a too short exposure to UV light and/or herbivory, resulting in a too low level of secondary metabolites to create a significant difference between the cultivation methods. Plants grown in temperature controlled sterile greenhouses are generally exposed to less abiotic (UV light) and biotic (herbivores) stress and might therefore produce fewer secondary metabolites. Li et al. (1996) for instance reported that a longer exposure period to direct sunlight promoted higher ginsenoside production in American ginseng plants. Nevertheless, our results show that while exposure to UV light and herbivores may increase the antimicrobial activity of leaf extracts for necklace orchids, plants grown indoors also possess antimicrobial activity. This result contradicts the common folk belief that medicinal orchids can only be harvested from the wild to maintain their potency. We therefore encourage cultivation of medicinal necklace orchids in order to prevent overexploitation and extinction of rare species in the wild.

The 70% ethanol leaf extracts of *C. fimbriata* showed in vitro antimicrobial activity against *S. aureus*, *B. cereus* and *Y. enterocolitica*, all known to cause gastrointestinal tract infections in humans. Activity was observed against both Gram-positive and Gram-negative bacteria, which indicates a broad spectrum of antimicrobial effects of leaf extracts of necklace orchids. The extracts were not able to inhibit growth of *E. coli* and *K. pneumoniae*. This can be explained by the fact that Gram-negative bacteria generally develop more resistance against synthetic antibiotics as compared with Gram-positive bacteria because they can more efficiently regulate genes involved in antibiotic drug resistance (Peleg and Hooper, 2010).

In contrast with the organ targeted EBDCS classification method, all the categories from the biological response (i.e. antimicrobial) classification method were found to be (highly) clustered. The biological response classification method can therefore be considered as more informative for bioprospecting. The biological response classification method also had a more scattered distribution of medicinal species on the phylogeny than the EBDCS classification method, covering a wider group of potential medicinal necklace orchid species by retrieving eight hot nodes as compared with the organ targeted EBDCS classification method, that only found three hot nodes. One of the eight hot nodes detected by the biological response classification method, but not by the organ targeted EBDCS classification method,

encompasses species of the necklace orchid genus *Bletilla*. Yang et al. (2012) successfully isolated bletilin A, bletilin B and other phenanthrenes from *Bletilla ochracea* tuber extracts that showed antibacterial activities against *S. aureus*, *S. epidermis* and *B. subtilis*. The fibrous roots and tubers from the *Bletilla striata* contain biphenanthrenes and stilbenoids, which possess antibacterial activity (Kovács et al., 2008; Qian et al., 2015), Additionally, dihydrophenanthrenes, phenanthrene, flavonoids, bibenzyl and phenolic compounds were isolated from entire plants of *B. formosana* by Lin et al (2005). These research findings support the results of our bioprospecting analyses and show that the biological response classification method is more effective in uncovering potential clades with high medicinal potential as compared with the EBDCS classification method.

Ethno-directed approaches in identifying plants traditionally used to treat specific diseases received significantly higher attention over the last decade as this method shows a relatively high success rate as compared to random plant screening programmes (Douwes et al., 2008; Siqueira et al., 2012). Plotting ethno-pharmacological data on a phylogenetic tree can be used as a time-efficient approach to discover potential new plant species with medicinal properties (Ernst et al., 2015), especially when a plant group is as large and diverse as the orchid family. We could only analyse 10% of all necklace orchid species for their medicinal properties. The reason for this was that while for some species with recorded medicinal use no DNA sequences were available, other species with known DNA sequences had not yet been investigated for their medicinal uses. We encourage more work on the ethnobotany and pharmacology of necklace orchids to increase species sampling. Especially species of the genera *Bletilla*, *Coelogyne* sect. *Bicellae*, sect. *Brachypterae*, sect. *Coelogyne*, sect. *Elatae*, sect. *Flaccidae*, sect. *Fuscescentes*, sect. *Hologyne*, sect. *Lawrenceana*, sect. *Lentiginosae*, sect. *Longifoliae*, sect. *Ocellatae*, sect. *Proliferae*, sect. *Ptychogyne*, sect. *Speciosae*, *Neogyna*, *Otochilus* and *Pholidota* sect. *Articulatae*, sect. *Chinenses*, sect. *Crinonia*, sect. *Pholidota* and sect. *Repentes* seem very promising for further research as these were identified to belong to hot node clades with high potency of antimicrobial activity.

5.5 Conclusions

We successfully employed bioprospecting to discover new necklace orchid species with antimicrobial activity. The traditional antimicrobial use of necklace orchids could be confirmed with bio-assays for leaf extracts prepared with 70% ethanol. Additionally, outdoor cultivation may result in increased antimicrobial activity, though this needs to be further explored. The biological response classification method was more effective in uncovering hot nodes leading to clades of species of necklace orchids with high antimicrobial potential as compared with the EBDCS classification method.

Supplementary data

DNA sequence alignments and bioinformatic scripts are available at Figshare, https://doi.org/10.6084/m9.figshare.13071893.v1

General discussion and conclusions

Chapter 6

General discussion and conclusions

In this chapter, I discuss the further steps needed to compliment the findings of this thesis and the work that must be continued to understand the (i) systematics, (ii) orchid-snail herbivory interactions, and (iii) bioprospecting of wild species of Necklace orchids in more detail.

6.1 Systematics

In the past two decades, orchid systematic studies have significantly advanced our understanding of the identification, diversification, and classification of the native species of Indonesia. Necklace orchids (Coelogyninae) have been intensively studied, especially the genera *Coelogyne* and *Dendrochilum* (Gravendeel et al., 2001; Clayton, 2002; George and George, 2011; Pedersen et al., 2019). However, it remained challenging to identify species of the overlooked genus *Glomera* because species description and distribution data were scattered throughout literature and natural history collections. In Chapter 2, I present a complete overview of the genus and species descriptions, distribution data, and illustrated interactive keys in a digital platform, Linnaeus, with a bilingual option (English-Bahasa). This method of presenting data is a major step forward, especially in the digital era, where it is essential to provide data that could be accessed anywhere and anytime by anyone. This platform provides easy access to hobbyists, naturalists, students, and researchers. The dual-language option makes it possible for Indonesian plant enthusiasts to read the species descriptions and associated information and increase knowledge about flower color and actual distribution patterns in the wild on popular social media such as Facebook, Observation.org, SmugMug, and WhatsApp.

During the research carried out for *Glomera*, I noticed that not all associated information of the specimens studied had been digitalized. Most

herbaria only digitalize a herbarium sheet and the information written on the label, but not any of the associated data hidden in exploration journals from the collector. This exploration journal could be in the archives of herbaria, other musea, or descendants of the researcher. In an exploration journal, a scientist writes more detailed information, like the color of flowers, altitude, local name, and possible uses, collecting dates, or even sketches or photographs of fresh plants and their natural surroundings. For my research, I could work with the diary of the Dutch explorer Gerard Marinus Versteeg (1876 - 1943), kindly made available by his grandson Anton Versteeg, that revealed important additional details about collecting dates and localities in New Guinea. Similar initiatives disclosing colonial cultural heritage such as '*Het geheugen van Nederland'* (geheugen.delpher.nl/nl) and '*Vele handen*' https://velehanden.nl/) will reveal many more metadata of natural history specimens and should be explored for this purpose much more often. Scientific explorations are time-consuming and costly, especially in the Indonesian archipelago due to scattered infrastructure. The involvement of the public in science, known as citizen science, is a solution where a collaboration between scientists and, in this case, amateur historians can help preparing botanical expeditions and increase the chances of finding rare orchid species flowering in the wild.

A major challenge for orchid identification is identifying non-flowering specimens as most identification keys are based on floral characters. Most orchid species require a very specific microclimate to produce flowers that is very hard to create in cultivation. Many wildly collected orchids, therefore, remain sterile and unidentified in cultivation. An accurate identification of species is essential for orchid conservation. DNA barcoding has been developed as an established tool for a rapid identification of species with short standard DNA sequences. This method is increasingly used to identify non-flowering orchids. However, DNA barcoding is usually carried out with fresh material, which is hardly available for rare and overlooked genera. In Chapter 3, I present a new approach: DNA barcoding of type specimens to identify non-flowering specimens of *Glomera*. Types usually remain untouched because the general view is that they should be left intact. However, sacrificing a small portion of a few leaves of types that have many can provide an essential DNA barcode for the identification of many sterile specimens. From a total of 84 specimens studied, we could identify 6 sterile

living collections with DNA barcodes generated from 32 types and 11 non-type specimens. To identify more sterile specimens, we urge for a more flexible policy regarding permission to generate DNA barcodes from additional type specimens.

A recent publication by Cámara-Leret et al. (2020) presented New Guinea as the world's island with the richest flora. Orchidaceae were listed as the plant family with the highest number of endemic species, 2,464, of which 144 are species from the genus that I studied in this thesis, *Glomera*. However, New Guinea lags behind other tropical regions, especially in taxonomic effort and explored area. More international collaboration with experts is needed to do joint scientific explorations to underexplored regions in New Guinea. This kind of collaboration is very important for young researchers to receive training and create a next generation of taxonomists and other local experts to safeguard the rapidly disappearing biodiversity of the Indonesian archipelago and beyond in Southeast Asia.

6.2 Orchid-snail herbivory interactions

Plant and herbivore interactions have been the subject of interest for many decades. Plants evolved various defenses to avoid consumption by herbivores, most notably biotic, physical, and chemical features. In contrast to the well-studied field of orchid pollination, only a few studies have been published on orchid-herbivory interactions, and most focus on insect herbivores (van Leeuwen, 1929; Subedi et al., 2011; Lev-Yadun and Ne'eman, 2012; Light and Macconaill, 2014) and myrmecochory, a mutualistic interaction with ants. The ants are attracted to elaiosomes, a lipid-rich part of orchid seeds. As a consequence, the ants transport the seeds to their nest, where the orchid can safely germinate and further develop under their protection (Dutta and Wetterer, 2008; Gegenbauer et al., 2012; Fisher, 2014).

In contrast, orchid-snail herbivores had not yet been studied. In Chapter 4, I investigated the epicuticular structures of four orchid species and their effect on snail attachment by measuring attachment forces using a centrifuge. I discovered that non-glandular and glandular trichomes and wax layers significantly reduce snail attachment. Cryo-Scanning Electron Microscopy, using liquid nitrogen rather than dehydration by ethanol and subsequent carbon dioxide fixation, could improve the study of wet epicuticular structures. A better understanding of the movements of epiphytic and terrestrial orchids,

caused by rain and wind, is also needed. Such knowledge could be obtained by using camera traps and measuring devices outside to record plant movements. The field data obtained can then be correlated with forces calculated in the laboratory for this thesis. In addition, cafeteria experiments should be carried out to further investigate the preference of snails for species of orchids with different protective structures next to trichomes and wax layers such as for instance, the membranous sheaths that subtend the flowers of *Glomera*. Knowledge obtained can be used to develop more eco-friendly protection of ex situ conserved orchids than the slug pesticides and other environmentally damaging controls currently employed.

6.3 Bioprospecting of wild orchids

Many Necklace orchid genera have traditional medicinal properties, such as *Coelogyne*, *Dendrochilum*, *Otochilus*, *Pholidota* and *Thunia* (Majumder et al., 2001; Wang et al., 2006; Moin et al., 2012; Marasini and Joshi, 2013; Shibu et al., 2013; Pant, 2014). However, none of these genera had been investigated ethnobotanically in a phylogenetic context yet. Bioprospecting has emerged as a time-efficient and systematic approach to discover potentially new medicinal plant species. To standardize the classification of ethnobotanical data from all countries, a system called Economic Botany Data Collection Standard (EBDCS) was established (Cook, 1995). In Chapter 5, I show that the organ-targeted EBDCS method is less evolutionary informative compared with the biological response method. A total of eight hot nodes were revealed using the biological responses method as compared to only three hot nodes with the EBDCS method. There is currently a lack of ethnobotanical information of Indonesian orchids. Bioprospecting with the biological response method might find additional clades containing medicinal Indonesian species when applied to other orchids used in traditional medicine in countries like China and India that have more established ethnobotanical practices than Indonesia. Possible examples are the Indonesian relatives of *Flickingeria macraei* (Lindl) Seidenf. and *Habenaria pectinata* D. Don. Compounds of these two orchid species are used to treat snakebites in India (Joshi et al., 2009). Both genera also occur in Indonesia, and phylogenetic prospecting could help detect Indonesian species with compounds that are useful for treating snake bites, also commonly occurring in Indonesia, that harbors ca. 450 species of snakes. An international Snakebite program was recently initiated by Naturalis Biodiversity Center and Leiden University to stimulate scientific knowledge exchange between science, government,

industry, and societal and humanitarian aid organizations to improve snakebite prevention and treatment. This initiative is supported by the World Health Organization and many countries like the Netherlands, Singapore, Costa Rica, Nigeria, the United Kingdom, and Denmark.

Nuclear Magnetic Resonance (NMR) could be applied to identify the bioactive compounds present in species from hot node clades. With the results obtained, public-private collaborations can be stimulated between botanical gardens, local communities, and companies like Martina Berto Tbk, producing cosmetics and herbal medicine that are committed to use local natural bioactive compounds in their products. Such initiatives are urgently needed to combat antibiotic-resistant microbes and new respiratory viruses like SARS-CoV-2 that cause COVID-19. *Eulophia nuda* Lindl and *Vanda tessellata* (Roxb.) Hook. ex G.Don have been used in India and Nepal to treat bronchitis (Vaidya et al., 2000; Singh and Duggal, 2009). These orchid species might contain a bioactive compound that could help relieve COVID-19 symptoms.

Finally, I discovered during the research carried out for this thesis that bioactive compounds are also produced when medicinal orchids are grown in cultivation. This discovery discards the persistent common belief that medicinal plants can only be collected from the wild, resulting in over-collecting and local extinction of many plant species. When exposed to more UV light and herbivores, the compounds of plants of *Coelogyne cristata* Lindl. and *C. fimbriata* Lindl. that I investigated showed an increased antimicrobial activity. Experiments conducted with more ecological variables should be conducted to develop sustainable and profitable cultivation of medicinal orchids.

Summaries

Summary

Indonesia is a tropical archipelago between Asia and Australia, and the Pacific and Indian Oceans with more than 17,000 islands. It is the second-highest biodiversity hotspot after Brazil for terrestrial flora and fauna and even the highest when combined with marine biodiversity. Indonesia is known to harbor 25% of all flowering plant species worldwide, of which 40% consists of Indonesian endemics. Recent surveys record a total of 7,622 orchid species distributed among the seven bio-regions of Indonesia. With approximately 7,622 species of orchids being present in Indonesia, more than 30% of all orchid species worldwide belong to the Indonesian flora. Over the past two centuries, research on Indonesian orchids gradually shifted from the discovery and description of new species to conservation and environmental studies.

This thesis reflects the gradual change in focus of research on Indonesian orchids as described in the previous paragraph. In Chapter 2, I present a multilingual interactive key, available online (http://glomera.linnaeus.naturalis.nl), that can be used on any web browser without the need for installing additional software. This key includes characters of 169 species of *Glomera*, a genus within the necklace orchids (Coelogyninae - Epidendroideae) not yet comprehensively treated in any recent field guide or web-based survey. With this key, species can be identified using a combination of vegetative and floristic characters in addition to distribution and ecology as a first step to further taxonomic revisions. In this chapter, I urge anyone with an interest in wild orchids in Southeast Asia to contribute new observations to update current information on the distribution of overlooked species to gain more insight into their conservation status.

In Chapter 3, I tackle a common challenge of ex situ orchid collections in botanic gardens. Most orchid species require very specific temperature, humidity, light levels and nutrient concentrations for flower induction and survival and therefore often remain sterile or die shortly after collection from the wild. This severely hampers the identification of such collections to species level as most identification keys require floral characters. DNA barcodes obtained from fertile type specimens in herbaria are a potential tool for fast species identification of sterile living collections, but only a few curators of herbaria allow destructive sampling of type specimens. I obtained permission to perform destructive DNA

extraction of a small number of the numerous leaves from type and non-type specimens of the poorly known necklace orchid genus *Glomera* preserved in the herbarium of Naturalis Biodiversity Center and Herbarium Bogoriense that were collected up to 194 years ago. I used four primer combinations to fully sequence the nrITS region of these specimens and fresh sterile living collections and obtained Sanger sequences for dried specimens and living collections. With the short sequences obtained, several sterile living collections could be identified to species level. Many of the living collections analyzed remained unnamed and are possibly new to science. My results show that DNA barcodes obtained from type material can provide reliable taxonomic information of sterile living collections. I propose a less rigorous policy regarding permission to generate DNA barcodes from additional type specimens to improve the identification of sterile specimens in living collections for better protection of poorly known orchid genera.

Once an orchid specimen with a known geographical origin in a living collection is identified to species level, it can for instance be used for the production of seeds and seedlings for reintroduction of a species into the wild if it is locally extinct. For such conservation efforts, it is important that plants in living collections remain healthy and alive for several years. In Chapter 4, I focus on one of the other challenges of a living orchid collection: suppressing herbivory. Intriguingly, some orchid species are less prone to herbivory than other species. Studies analysing protection of orchids against herbivores require an integrative approach, combining anatomical studies of epicuticular trichomes and waxes with behavioural experiments of the herbivores. In this study, I show for the first time that the terrestrial orchids *Orchis mascula* and *Calanthe triplicata*, the first species deciduous and the second evergreen, protect themselves very differently against attachment of herbivorous land snails than two evergreen epiphytic orchids *Dendrochilum pallidiflavens* and *Trichotosia ferox*, using histochemistry, ‘cafetaria’ and centrifuge experiments. Size and ornamentation of wax layers and density and histochemistry of epicuticular glandular and non-glandular trichomes on the orchid leaves were assessed with Light Microscopy, Scanning Electron Microscopy and Transmission Electron Microscopy. Total forces needed to detach two differently shaped snail species, *Subulina octona* and *Pleurodonte isabella*, were measured using a turntable equipped with a synchronized strobe. Snails were placed in two positions, either perpendicular or parallel to the main veins on

the orchid leaves and on the adaxial (=upper) or abaxial (=lower) side. *Subulina octona* snails were fed with young leaves of *Calanthe triplicata* leaves, of which the hairs had been removed with a lighter in half of the leaves beforehand. The percentage of leaves consumed by the snails was significantly higher for the leaves without hairs. The results obtained provide two other insights. First of all, a perpendicular or parallel position of the snails to the main veins significantly affects the performance of the smaller species tested. Secondly, snails come off significantly faster on sides covered by a thick wax layer or high density of lignin filled non-glandular epicuticular trichomes. This study highlights the importance of histology in combination with behaviour and attachment force experiments for obtaining a better understanding of the defense mechanisms employed by different species of epiphytic and terrestrial orchids to deter herbivorous snails. With this knowledge, orchid individuals with an optimal combination of protective traits, such as a thick wax layer or long non-glandular lignified trichomes, can be selected for future breeding and reintroduction programs.

Another challenge of maintaining collections of living specimens of orchids is the many hours of manual labor required to keep the plants alive. It is a lot of work to daily water, fertilize and/or shade each individual plant according to its own specific needs and keep it protected from herbivores. This work cannot be robotized. In Chapter 5, I focus on a potential extra source of income that could be generated for paying sufficient staff to maintain labor intensive living orchid collections. Extra revenues could be generated by setting up innovative public-private collaborations that are making use of bio-active compounds harvested from orchids. Necklace orchids have been used for traditional medicine practices for centuries. Previously carried out bioassays on a subset of unrelated species showed promising antimicrobial, anti-inflammatory, and anti-oxidant bioactivity, providing experimental proof for medicinal properties as recorded in traditional uses. However, none of these species had been investigated ethnobotanically in a phylogenetic context, yet, at the onset of my PhD project. For my thesis, I carried out comparative bioprospecting of a group of wild orchids using EBDCS (the Economic Botany Data Collection Standards) organ targeted and biological response methods. Traditional medicinal use was recorded from books and journals. Bioassays with a selection of human pathogen microbes were carried out in triplicate on various extractants of leaves and pseudobulbs of *Coelogyne*

cristata and *C. fimbriata* plants cultivated indoors or outdoors. A molecular phylogeny of Coelogyninae based on nuclear ribosomal ITS and plastid matK DNA sequences obtained from a total of 148 species was reconstructed with Maximum Parsimony (MP), Maximum Likelihood (ML) and Bayesian Inference using MrBayes. Bioprospecting comparison of EBDCS and biological response was carried out using customized R scripts. For a total of 28 necklace orchid species, traditional uses could be compiled, encompassing 19 organ-targeted categories and one biological response category with three different character states. Ethanolic extracts obtained from leaves of *C. fimbriata* were found to inhibit growth of *Bacillus cereus*, *Staphylococcus aureus*, and *Yersenia enterocollitica* and confirmed traditional antimicrobial uses recorded in the literature. Leaf extracts were found to have similiar antimicrobial properties for plants cultivated outdoors and indoors. Three hot nodes with high potency for antimicrobial activities were detected for the EBDCS organ targeted classification method whereas eight hot nodes were detected for the biological response classification method. I conclude that the biological response classification method is more effective in uncovering hot nodes leading to clades of species with high medicinal potential as compared with the EBDCS classification method. I recommend to apply this method to other subtribes to further explore the potential economic uses of bio-active compounds of Indonesian orchid species.

Samenvatting

Indonesië bestaat uit 17,000 eilanden, die tussen het vasteland van Azië en Australië liggen in de Pacifische en Indische oceaan. Het land bevat na Brazilië het hoogste aantal planten- en dierensoorten ter wereld, en zelfs de meeste soorten als ook al het mariene leven binnen de territoriale wateren wordt meegeteld. Van alle landplanten komt een kwart alleen in Indonesië voor. Met meer dan 7,600 soorten orchideeën, verspreid over de zeven fytogeografische regio's, draagt de Indonesische flora voor meer dan een derde bij aan alle soorten van deze plantenfamilie. Gedurende de afgelopen twee eeuwen veranderde het onderzoek aan Indonesische orchideeënsoorten langzaam van het ontdekken en beschrijven van nieuwe soorten in het behouden van soorten en het ontdekken van interacties van soorten met hun omgeving.

Dit proefschrift getuigt van bovenbeschreven verandering van focus in het onderzoek aan Indonesische orchideeënsoorten. In hoofdstuk 2 presenteer ik een interactieve determinatiesleutel in twee talen (http://glomera.linnaeus.naturalis.nl), die online gebruikt kan worden. Naast een web browser hoeven voor deze sleutel geen extra programma's geïnstalleerd te worden. De determinatiesleutel bevat kenmerken van 169 soorten van *Glomera*, een genus binnen de Coelogyninae (Epidendroideae). Van deze groep orchideeën was nog geen compleet overzicht beschikbaar. Met de determinatiesleutel kunnen soorten op naam gebracht worden met een combinatie van kenmerken van het rhizoom, de stengels, bladeren, pseudobulben en bloemen, aangevuld met gegevens over geografische verspreiding en ecologie. In dit hoofdstuk vraag ik iedereen, met een passie voor wilde orchideeën uit Zuidoost Azië, om de sleutel verder te helpen verbeteren met aanvullingen in bijvoorbeeld verspreidingsdata. Zo kunnen we een vollediger beeld krijgen van de zeldzaamheid van soorten en het uitsterven van die soorten helpen voorkomen.

In hoofdstuk 3 presenteer ik een oplossing voor een belangrijke uitdaging van ex situ orchideeëncollecties in botanische tuinen: het niet in bloei komen van in het wild verzamelde planten. Veel orchideeënsoorten komen pas in bloei bij een hele specifieke combinatie van temperatuur, vochtigheid, licht en nutriëntenconcentraties. Die combinatie is vaak heel moeilijk na te bootsen in een kas. Veel in het wild verzamelde planten gaan dan ook dood of komen nooit

in bloei in een levende collectie. Zonder bloemen zijn veel orchideeënsoorten lastig op naam te brengen omdat de meeste determinatiesleutels bloemkenmerken bevatten. DNA barcodes, gegenereerd uit op naam gebrachte typecollecties met bloemen, kunnen in dit geval het identificeren van niet bloeiende planten in levende collecties helpen bespoedigen. Collectiebeheerders geven echter meestal geen toestemming voor het destructief bemonsteren van type materiaal. De meeste herbaria reageerden dan ook negatief op een dergelijk verzoek maar uiteindelijk kreeg ik gelukkig toestemming om wat kleine stukjes blad te mogen bemonsteren van type collecties van verschillende *Glomera* soorten uit het herbarium van Naturalis Biodiversity Center en het Herbarium Bogoriense, en wel van herbariumexemplaren met relatief veel bladmateriaal. Het oudste exemplaar is 194 jaar geleden verzameld. Uit die stukjes blad heb ik DNA geëxtraheerd en de nrITS regio's geamplificeerd en gesequenced met behulp van vier primer combinaties. Door de gegenereerde Sanger sequenties te vergelijken met DNA barcodes van levend maar niet bloeiend materiaal, konden enkele planten uit de collecties van de Hortus botanicus in Leiden en Kebun Raya in Bogor met een zekerheid van 100% op naam gebracht worden. Voor een aantal andere exemplaren met afwijkende DNA barcodes geldt wellicht dat het om nog niet beschreven soorten gaat. Met de door mij toegepaste DNA barcodingmethode van type collecties kunnen niet bloeiende planten dus op naam gebracht worden. Ik pleit dan ook voor een versoepeling van het huidige beleid ten aanzien van destructieve bemonstering van type exemplaren. Immers, met DNA barcodes van type collecties kan meer inzicht verkregen worden in de huidige verspreiding van zeldzame soorten zodat ze beter beschermd kunnen worden.

Als een orchidee met vindplaatsgegevens in een levende collectie éénmaal op naam is gebracht kan zo'n plant gebruikt worden voor de productie van zaden en kiemplanten voor herintroductie in de natuur als de soort ter plekke dreigt uit te sterven of al uitgestorven is. Voor dit type herstelbeheer zijn langlevende en gezonde planten nodig in ex situ collecties want het duurt een aantal jaar voordat een geherintroduceerde populatie het verder redt in situ zonder het bijplanten van extra kiemplanten. In hoofdstuk 4 presenteer ik een oplossing voor een andere uitdaging van levende orchideeëncollecties, namelijk het overleven van herbivorie. Tijdens veldwerk in Ambon viel het mij op dat sommige orchideeën minder worden gegeten door herbivoren dan andere soorten. Om uit te zoeken hoe orchideeën

zich tegen herbivoren beschermen is een multidisciplinaire aanpak nodig. Ik heb daarom anatomisch en histologisch onderzoek aan haren en waslaagjes op de epidermis gecombineerd met gedragsexperimenten van herbivoren. In dit onderzoek laat ik voor het eerst zien dat de terrestrische orchideeënsoorten *Orchis mascula* en *Calanthe triplicata*, de eerste bladverliezend, de tweede niet, zich heel anders tegen herbivore landslakjes verdedigen dan de twee niet bladverliezende epifytische soorten *Dendrochilum pallidiflavens* en *Trichotosia ferox*. De waslaag en haren van deze orchideeënsoorten zijn onderzocht met zowel lichtmicroscopie, als scanning en transmissie elektronenmicroscopie. In een 'cafeteria' experiment werd jong blad van *Calanthe triplicata* aangeboden aan de herbivore slakkensoort *Subulina octona*. De haren op dit blad waren in de helft van het aangeboden materiaal van te voren met een aansteker weggebrand. De schade, veroorzaakt door herbivorie, was aanzienlijk groter in het bladmateriaal zonder haren. Krachten, nodig om *Subulina octona* en *Pleurodonte isabella*, twee slakkensoorten met respectievelijk veel en weinig windingen in de huisjes, van orchideeënblad te verwijderen, zijn vervolgens vastgesteld met een draaitafel met gesynchroniseerde belichting. Slakken werden evenwijdig of parallel aan de primaire nerven op een vers geplukt orchideeënblad gezet, en dit zowel op de onderkant als op de bovenkant van het blad. Vervolgens werd de centrifuge aangezet en steeds sneller rondgedraaid totdat de slak losliet. De gemeten waardes lieten zien dat de microstructuur van een waslaag bescherming biedt tegen de kleinere slakkensoort. Verder was aanmerkelijk minder kracht nodig om slakken te verwijderen van blad bedekt met een waslaag of een hoge dichtheid aan haren gevuld met lignine. Op basis van deze resultaten adviseer ik om exemplaren van de onderzochte soorten te selecteren met een dikkere waslaag of dichtere beharing voor betere bescherming tegen herbivore slakken in veredelings- en herintroductieprogramma's.

Een laatste uitdaging van levende orchideeëncollecties betreft de vele uren fysieke arbeid die jaarlijks nodig zijn om planten in leven te houden. Iedere plant heeft een specifieke combinatie van water, bemesting, licht en schaduw nodig, naast een regelmatige controle op herbivorenschade. Dit werk kan niet geautomatiseerd worden. In hoofdstuk 5 presenteer ik een potentiële extra bron van inkomsten om de onderhoudskosten van arbeidsintensieve levende plantencollecties deels mee te bekostigen. Extra inkomsten kunnen worden

gegenereerd uit een vernieuwende publiek-private samenwerking op het gebied van bioactieve inhoudsstoffen uit orchideeën. Coelogyninae orchideeën worden al eeuwenlang gebruikt als traditioneel medicijn. Eerder uitgevoerde bioassays met slechts een paar soorten lieten al veelbelovende resultaten zien. Extracten van deze orchideeën hebben antimicrobiële en koortswerende eigenschappen en kunnen als antioxidant functioneren. Geen van deze soorten was echter al ethnobotanisch onderzocht in een fylogenetische context. Ik heb dat laatste gedaan en daarbij twee indelingen met elkaar vergeleken: de op organen gebaseerde Economische Botanie Data Collectie Standaard (EBDCS) en de biologische responsmethode. Traditioneel medicinaal gebruik van Coelogyninae werd eerst uit de literatuur verzameld. Vervolgens heb ik bioassays uitgevoerd met een selectie aan antibiotica resistente lijnen van humaan pathogene microbes en extracten verkregen uit blad en pseudobulben van twee soorten Coelogyninae: *Coelogyne cristata* en *C. fimbriata*. Er zijn extracten gebruikt van planten die onder glas gekweekt werden en van planten die gekweekt werden in de buitenlucht. Een moleculaire fylogenie van 148 soorten Coelogyninae, gebaseerd op DNA sequenties van nucleaire rITS en chloroplast matK markers, is gebruikt om het traditionele medicinale gebruik in een fylogenetische context te plaatsen. Voor 28 soorten Coelogyninae is het traditioneel gebruik ingedeeld volgens de EBDCS methode, met 19 categorieën, en de biologische responsmethode, met drie categorieën. Met behulp van ethanol geëxtraheerde inhoudsstoffen van het blad van *Coelogyne cristata* en *C. fimbriata* remden de groei van *Bacillus cereus*, *Staphylococcus aureus* en *Yersenia enterocollitica*. Dit bevestigde het traditioneel medicinaal gebruik zoals beschreven in de literatuur. Extracten van in openlucht gekweekte planten bleken een sterkere antimicrobiële werking te hebben dan extracten van onder glas gekweekte planten. Met de EBDCS methode werden slechts drie clades met een hoge kans op soorten met antimicrobiële eigenschappen ontdekt, terwijl met de biologische responsmethode maar liefst acht clades werden gevonden. Mijn conclusie is dan ook dat de laatste methode effectiever is. Mijn advies is om de biologische responsmethode nu ook toe te passen op andere orchideeëngroepen met veel vertegenwoordigers in Indonesië. Zo kunnen nieuwe toepassingen gevonden worden voor de bioactieve inhoudsstoffen van deze soorten om hiermee de kosten van het onderhoud van een levende collectie deels terug te verdienen.

Ringkasan

Indonesia merupakan negara kepulauan tropis yang memiliki 17.000 pulau serta terletak diantara dua benua (Asia dan Australia) dan dua samudera (Samudera Pasifik dan Samudera Hindia) . Indonesia merupakan pusat biodiversitas kedua terbesar setelah Brazil untuk tumbuhan dan hewan darat, bahkan menjadi yang tertinggi jika digabung dengan biodiversitas laut. Indonesia merupakan tempat tumbuh dari 25% dari total tumbuhan berbunga di dunia, dimana 40% diantaranya merupakan endemik Indonesia. Berdasarkan survey terkini diketahui ada 7,622 jenis anggrek yang tersebar pada 7 bioregion di Indonesia. Dengan jumlah ini lebih dari 30% dari jenis anggrek di dunia merupakan tumbuhan asli Indonesia. Lebih dari dua puluh abad, riset anggrek Indonesia kini beralih dari penemuan dan deksripsi jenis baru menuju ke konservasi dan studi lingkungan.

Tesis ini menggambarkan peralihan fokus riset anggrek Indonesia sebagaimana dijelaskan pada paragraf sebelumnya. Pada Bab 2, saya menyajikan kunci interaktif multibahasa, tersedia secara daring (http://glomera.linnaeus.naturalis.nl), yang dapat digunakan pada berbagai halaman peramban tanpa harus mengunduh perangkat lunak apapun. Kunci identifikasi ini berisi karakter dari 169 jenis *Glomera*, marga dalam anggrek kalung (Coelogyninae – Epidendroideae) yang belum diamati secara komprehensif pada panduan lapangan terbaru atau survei berbasis daring. Dengan kunci identifikasi ini, anggrek dapat diidentifikasi sampai tingkat jenis dengan menggabungkan kombinasi karakter vegetatif, perbungaan, distribusi dan ekologi sebagai langkah awal untuk revisi taksonomi lebih lanjut. Dalam bab ini, saya menghimbau siapa pun yang berkepentingan dengan anggrek liar di Asia Tenggara untuk menyumbangkan pengamatan terbaru guna memperbaharui informasi terkini tentang sebaran jenis yang belum banyak dikenal ini sehingga mendapatkan wawasan lebih banyak tentang status konservasi jenis tersebut.

Dalam Bab 3, saya membahas tentang tantangan umum koleksi anggrek di luar habitatnya di kebun raya. Sebagian besar jenis anggrek memerlukan suhu, kelembapan, tingkat cahaya dan konsentrasi nutrisi yang sangat spesifik untuk memicu pembungaan dan mempertahankan agar tumbuhan ini tetap hidup, bahkan banyak spesies yang tetap hidup tanpa bunga atau mati beberapa saat setelah dikoleksi dari hutan. Hal ini sangat menghambat identifikasi hingga

tingkat jenis untuk koleksi tersebut karena kunci identifikasi sangat memerlukan karakter bunga. Barkoding DNA yang diperoleh dari spesimen tipe yang mempunyai bunga merupakan alat potensial untuk proses identifikasi yang lebih cepat untuk koleksi hidup yang tidak mempunyai bunga, akan tetapi hanya sedikit kurator herbaria yang mengizinkan pengambilan sampel herbarium tipe. Saya mendapatkan izin untuk melakukan ekstraksi DNA dari sejumlah kecil daun dari specimen tipe dan non-tipe dari marga yang jarang diketahui dari anggrek kalung, *Glomera* Blume yang diawetkan di herbarium Naturalis Biodiversity Center dan Herbarium Bogoriense yang dikoleksi sejak 194 tahun lalu. Saya menggunakan empat kombinasi primer untuk mengurutkan genom inti ITS dari spesimen tipe dan koleksi hidup tanpa bunga dan mendapatkan sekuen Sanger untuk spesimen kering dan koleksi hidup. Dengan sekuen pendek yang diperoleh, beberapa koleksi hidup tanpa bunga dapat diidentifikasi hingga tingkat jenis. Banyak dari koleksi hidup yang dianalisis belum dapat diidentifikasi dan kemungkinan merupakan jenis baru. Hasil ini menunjukkan bahwa barkoding DNA yang diperoleh dari spesimen tipe dapat memberikan informasi taksonomi yang dapat digunakan untuk mengidentifikasi untuk koleksi hidup tanpa bunga. Saya mengusulkan kebijakan yang fleksibel mengenai izin untuk menghasilkan barkoding DNA dari spesimen tipe untuk dapat mengidentifikasi spesimen tanpa bunga dalam koleksi hidup sehingga akan ada perlindungan lebih baik untuk marga anggrek yang tidak banyak diketahui.

Setelah spesimen anggrek yang diketahui sebaran geografisnya dalam koleksi hidup dapat diidentifikasi hingga level jenis, hasil ini dapat digunakan untuk memproduksi biji dan benih untuk reintroduksi dari jenis tesebut ke alam liar jika spesies tersebut punah secara lokal. Dan agar upaya pelestarian ini tetap berlanjut maka tumbuhan dalam koleksi harus tetap sehat dan hidup selama bertahun-tahun. Dalam Bab 4, saya fokus pada tantangan lain dari koleksi anggrek: mencegah hama herbivora. Fakta yang menarik di lapangan adalah jenis anggrek epifit lebih tidak rentan terhadap hama herbivora dibandingkan jenis anggrek terestrial. Studi untuk menganalisis ketahanan anggrek terhadap hama herbivora memerlukan pendekatan yang terintegrasi, yang menggabungkan studi anatomi trikoma epikutikular dan lapisan lilin dengan eksperimen perilaku herbivora. Dalam studi ini, pertama kalinya saya menunjukkan bahwa anggrek terrestrial *Orchis mascula* dan *Calanthe triplicata*, spesies pertama gugur daun dan kedua

hijau sepanjang tahun, mempunyai sistem perlindungan yang berbeda terhadap herbivora siput tanah dibandingkan dengan dua jenis anggrek epifit hijau sepanjang tahun *Dendrochilum pallidiflavens* dan *Trichotosia ferox*, dengan menggunakan histokimia dan eksperimen sentrifuse. Ketebalan dari lapisan lilin serta kepadatan dan histokimia trikoma kelenjar dan non-kelenjar pada epikutikular daun anggrek diamati menggunakan mikroskop cahaya, mikroskop pemindai elektron dan mikroskop transmisi elektron. Total daya yang diperlukan untuk melepaskan dua jenis siput yang berbentuk berbeda, *Subulina octona* dan *Pleurodonte isabella* diukur menggunakan mesin sentrifuse yang dilengkapi oleh strobe tersinkronisasi. Siput ditempatkan dalam dua sisi, tegak lurus atau sejajar dengan urat utama pada daun anggrek, dan pada sisi adaxial (bagian atas) atau abaxial (bagian bawah). Hasil yang diperoleh memberikan dua fakta baru. Pertama, posisi siput tegak lurus atau sejajar dengan urat utama mempengaruhi kinerja spesies lebih kecil yang diuji. Kedua siput terlepas lebih cepat pada sisi yang ditutup oleh lapisan lilin tebal atau trikoma non-kelenjar epikutikular dengan kepadatan tinggi yang mengandung lignin. Studi ini menyoroti pentingnya histologi dan kombinasi dengan eksperimen daya perlekatan untuk mendapatkan pemahaman yang lebih baik tentang mekanisme pertahanan yang digunakan berbagai jenis anggrek epifit dan terestrial untuk mencegah hama siput herbivora. Dengan pengetahuan ini, individu anggrek dengan kombinasi sifat protektif yang optimal seperti lapisan lilin tebal atau trikoma non-kelenjar yang mengandung lignin dan panjang dapat dipilih untuk program pemuliaan dan reintroduksi di masa mendatang.

Tantangan lain dalam memelihara koleksi spesimen anggrek adalah banyaknya waktu yang dibutuhkan untuk menjaga agar tumbuhan tetap hidup. Pekerjaan tersebut termasuk menyiram setiap hari, memberi pupuk dan memberi naungan pada setiap jenis anggrek sesuai dengan kebutuhan spesifiknya dan menjaganya tetap terlindungi dari hama herbivora. Pekerjaan ini hanya bisa dilakukan secara manual tanpa bantuan robot. Dalam Bab 5, saya fokus pada potensi sumber pendapatan tambahan yang dapat dihasilkan untuk membayar staff untuk memelihara koleksi anggrek yang membutuhkan banyak tenaga. Pendapatan tambahan dapat dihasilkan dengan membentuk kolaborasi inovatif antara publik dan swasta dengan memanfaatkan senyawa bioaktif yang dipanen dari anggrek. Anggrek kalung telah banyak digunakan dalam praktik pengobatan tradisional selama berabad-abad. Bioassay yang dilakukan sebelumnya pada subset spesies

yang tidak terkait menunjukkan bioaktivitas antimikroba, anti peradangan dan antioksidan yang menjanjikan, dan hasil ini memberikan bukti eksperimental untuk sifat obat yang tercatat dalam pengobatan tradisional. Namun tidak satupun dari jenis ini telah diteliti secara etnobotani dalam konteks filogenetik, setidaknya pada saat saya memulai proyek PhD ini. Dalam tesis saya, saya melakukan bioprospeksi komparatif pada sekelompok anggrek liar menggunakan metode respon biologis dan target organ EBDCS (Standar Pengumpulan Data Botani Ekonomi). Penggunaan anggrek dalam pengobatan tradisional tercatat dalam buku dan jurnal. Bioassay menggunakan mikroba pathogen manusia dilakukan dengan 3 kali pengulangan untuk ekstrak yang didapatkan dari berbagai ekstraktan yang diperoleh dari daun dan pseudobulb anggrek jenis *Coelogyne cristata* dan *C. fimbriata* yang dibudidayakan di dalam maupun di luar rumah kaca. Filogeni molekular Coelogyninae berdasarkan sekuens DNA ribosom inti ITS dan kloroplas matK yang diperoleh dari total 148 jenis direkonstruksi dengan Maximum Parsimony (MP), Maximum Likelihood (ML) dan Bayesian Inference menggunakan MrBayes. Perbandingan bioprospekting EBDCS dan respon biologis dilakukan dengan menggunakan skrip R yang telah disesuaikan. Total 28 jenis anggrek kalung yang dapat diperoleh informasi penggunaan tradisional yang mencakup 19 kategori target organ dan 1 kategori respon biologis dengan 3 status karakter yang berbeda. Ekstrak etanolik yang diperoleh dari daun *C. fimbriata* ditemukan dapat menghambat pertumbuhan *Bacillus cereus*, *Staphylococcus aureus*, dan *Yersenia enterocollitica* dan hasil ini mengkonfirmasi efek antimikroba yang telah tercatat dalam literatur. Ekstrak daun memiliki sifat antimikroba yang sama untuk tanaman yang dibudidayakan di luar dan di dalam rumah kaca. Tiga kluster dengan potensi tinggi untuk aktivitas antimikroba terdeteksi untuk metode klasifikasi target organ EBDCS sedangkan delapan kluster terdeteksi untuk metode klasifikasi respons biologis. Saya menyimpulkan bahwa metode klasifikasi respons biologis lebih efektif dalam mengungkap kluster yang mengarah ke kluster spesies dengan potensi tinggi sebagai obat dibandingkan dengan metode klasifikasi EBDCS. Saya merekomendasikan untuk menerapkan metode ini pada marga lain untuk lebih mengeksplorasi potensi ekonomi senyawa bioaktif anggrek Indonesia.

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Curriculum Vitae

Richa Kusuma Wati was born on August 17th, 1984, in Malang, Indonesia, and grew up in East Java Province. Her passion for chemistry in high school led her to study Food Technology at Brawijaya University, Indonesia, where she obtained her bachelor's degree in 2006. Just before graduation, she was granted a double-degree scholarship from KEMDIKBUD. She completed her M.Sc in 2009 from Brawijaya University, Indonesia, and Mae Fah Luang University, Thailand, with publishing three papers about the purification of trypsin inhibitor from legumes to preserve fish products. After her graduation, she was accepted in Bogor Botanic Gardens-LIPI as a researcher. For her first task she was involved with orchid collections in the gardens. She met her mentor, Sofi Mursidawati, and worked together to manage the orchid collections. In 2015, they published an orchid catalog of the garden, fifteen years after the publication of the first catalog. During this time, she became interested in orchids and this led her to study orchids for her PhD project. Since November 2015, after receiving a scholarship from LPDP, she started her PhD research at Naturalis Biodiversity Center and Leiden University under the supervision of Prof. Barbara Gravendeel and Prof. Erik Smets. She will continue to work as an orchid researcher in Bogor Botanic Gardens. Her main future research projects will be focused on orchid ex-situ conservation, saving the endangered orchid species of Indonesia by applying genomics, taxonomy, systematics, and bio-prospecting. Richa is also interested in understanding orchid-pollinator-herbivore interactions.

List of Publications

Wati, R.K., de Graaf, E., Bogarin, D., Heijungs, R., van Vugt, R.R., Smets, E.F. & B.Gravendeel. 2021.Antimicrobial activity of necklace orchids is phylogenetically clustered and can be predicted with a biological response method. Frontiers in Pharmacology, section Ethnopharmacology (in press).

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PE&RC Training and Education Statement

With the training and education activities listed below the PhD candidate has complied with the requirements set by the C.T. de Wit Graduate School for Production Ecology and Resource Conservation (PE&RC) which comprises of a minimum total of 32 ECTS (= 22 weeks of activities)

Review of literature (4.5 ECTS)

- Phylogenetic prospecting of Indonesian Coelogyninae (Orchidaceae) used as traditional antimicrobials

Post-graduate courses (7.8 ECTS)

- Metabolomics; IBL (2016)
- Phylogenetics; Wageningen University (2018)
- Introduction to ArcGIS; Leiden University & Naturalis (2018)

Laboratory training and working visits (0.3 ECTS)

- GGO Training for ML2 lab; Leiden University (2016)

Competence strengthening / skills courses (1.5 ECTS)

- Time management, self- management; Leiden University (2017)
- Effective communication; Leiden University (2017)
- Communication in science; Leiden University (2017)

Scientific integrity / ethics in science activity (0.3 ECTS)

- On being a scientist; Leiden University (2016)

Discussion groups / local seminars / other scientific meetings (6.6 ECTS)

- Learning from nature, learning from our ancestors, from tradition to evidence-based medicines (2016)
- Endless forms (2016-2019)

International symposia, workshops and conferences (5.1 ECTS)

- 18th European orchid council conference and exhibition; Paris (2018)
- 2nd Conference of the Netherlands Society for Evolutionary Biology (NLSEB); Wageningen (2019)
- 7th International orchid conservation congress; Royal Botanic Garden Kew (2019)

Societally relevant exposure (1 ECTS)

- Presentation; WEO (Werkgroep Europese Orchideeën), Maarn (2019)

Lecturing / supervision of practicals / tutorials (0.6 ECTS)

-Biology of Orchids

BSc thesis supervision (12 ECTS)

- Medicinal properties of Indonesian Coelogyne
- Antimicrobial properties of Indonesian Coelogyninae on antibiotic-resistant bacteria
- Bioprospection of Indonesian medicinal orchids (Coelogyninae) seems promising for finding potential new species for alternative drug discovery
- The epicuticular properties of orchids and their effect for snail herbivory activity


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