ГОДИШНИК НА СОФИЙСКИЯ УНИВЕРСИТЕТ „СВ. КЛИМЕНТ ОХРИДСКИ“
БИОЛОГИЧЕСКИ ФАКУЛТЕТ
Книга 2 - Ботаника
Том 106, 2022

ANNUAL OF SOFIA UNIVERSITY “ST. KLIMENT OHRIDSKI”
FACULTY OF BIOLOGY
Book 2 - Botany
Volume 106, 2022

REVIEW ON BIOLOGICAL AND BIOTECHNOLOGICAL CHARACTERISTICS OF THE TERRESTRIAL ORCHID *LUDISIA DISCOLOR*

NIKOLAY P. KRUMOV1, VIKTORIA A. HRISTOVA2, GEORGI N. BONCHEV3, ANELY M. NEDELCHEVA4, ALEXANDER A. TOMOV1 & MIROSLAVA K. ZHIPONOVA1*

Department o f Plant Physiology, Faculty o f Biology, Sofia University “St. Kliment Ohridski”, 8 Dragan Tsankov blvd., 1164 Sofia, Bulgaria
2Department o f Botany, Faculty o f Biology, Sofia University “St. Kliment Ohridski”, 8 Dragan Tsankov blvd., 1164 Sofia, Bulgaria
3Department o f Molecular Biology and Genetics, Institute o f Plant Physiology and Genetics, Bulgarian Academy o f Sciences, 1113 Sofia, Bulgaria
4Department o f Organic Chemistry and Pharmacognosy, Faculty o f Chemistry and Pharmacy, Sofia University “St. Kliment Ohridski”, 1 James Bourchier blvd.,1164 Sofia, Bulgaria

Abstract. The present paper presents a review of the distribution, taxonomy, ecology, biochemical properties and usage of the orchid *Ludisia discolor.*

Keywords: DNA barcoding, jewel orchid, micropropagation, mycorrhiza, photosynthesis, phytochemical potential

* *corresponding author:* M. K. Zhiponova, Department of Plant Physiology, Faculty of Biology, Sofia University “St. Kliment Ohridski”, 8 Dragan Tsankov blvd., 1164 Sofia, Bulgaria; zhiponova@biofac.uni-sofia.bg

INTRODUCTION

Ludisia discolor (Ker-Gawl.) A. Rich. is a species of terrestrial orchid from subfamily Orchidoideae originating in parts of the Asian continent but commonly grown as an ornamental plant in many countries outside of its natural range. Similarly to many of its closest relatives, it is often described as a jewel orchid, a nickname applied due to the characteristic coloration of its leaves which give the plant its attractive appearance. Taxonomic relationships among the members of the jewel orchid group have been established through molecular approaches, such as DNA barcoding serving as a valuable tool for quick species identification of morphologically similar individuals. The overall morphology of *L. discolor* is a product of the orchid's natural environment which involves shady and forested habitats with reduced levels of sunlight. Hence, the plant requires special adaptations for better efficiency of the photosynthetic process. As a member of the orchid family, it forms mycorrhizal associations with different species of fungi, many of which remain largely undiscovered so far. Another important feature of *L. discolor* is that it can produce a large variety of phytochemicals, especially plant glycosides such as kinsenoside, goodyeroside and gastrodin among others. Many of these compounds have been studied for their potential pharmacological activity both in vitro and in vivo thus giving a promising perspective for the development of new sets of drugs targeting various medical conditions. This finding sparks an interest in the plant's secondary metabolism since many of the biochemical processes responsible to produce the orchid's bioactive chemicals have not been completely elucidated yet. These potential applications as well as the demands of the floral industry stimulate the research in the mass propagation of *L. discolor*. Vegetative reproduction is achieved relatively easy and explant cultures involving sterile conditions and in vitro techniques have been successfully established.

RESULTS

Distribution - natural and by trade

Ludisia discolor (Ker-Gawl.) A. Rich. is distributed in southeastern Asia, as its native geographical range spans countries in the mainland such as southern China, northeastern India, Thailand, Vietnam, Laos and Myanmar but also extends into various islands off the shore which includes territories of Indonesia, Malaysia and the Philippines (**Kew Science** 2020) (Fig. 1). These regions are characterized by relatively stable warm climates with high levels of air moisture. *L. discolor* is often found growing in swampy habitats or in the understory of tropical and subtropical forests where the degree of illumination is relatively low (**Averyanov et al.** 2003).

Even though the orchid is widespread in its native geographical range and is common in cultivation, some of its populations have been decreasing. This is mostly due to habitat loss as well as to beliefs in folk medicine which attribute various

14

Fig. 1. Map of the natural distribution of *L. discolor*.

medicinal properties to the plant. As a result, uncontrolled gathering of specimens from their natural environment and trade of the plant have been often (Teoh 2016). *L. discolor* is commonly grown as an ornamental plant in many countries outside of its natural range (Fig. 2). *L. discolor* is the principal orchid species accounting for 45% of live plant trade mostly exported by Amazon countries and imported by the Netherlands (Sinovaset al. 2017).

Taxonomic affiliation

Ludisia is generally considered a monotypic genus with *L. discolor* being the only known member (Chen et al. 2019). *Ludisia* belongs to the Orchidoideae subfamily of orchids which spans most of the terrestrial members of Orchidaceae. It is placed in Cranichideae tribe, which is overall considered monophyletic and further classified in the subtribe of Goodyerinae (Chase et al. 2015; Chen et al. 2019). Goodyerinae

Fig. 2. *L.* discolorplant as a houseplant: **a** - plant view; **b** - inflorescence; **c** - young leaves. Scale bars are shown.

15

contains about 35 genera with many of the species colloquially referred to as “jewel orchids” for reasons like those for *Ludisia*, namely their variegated foliage, which provides them with a high ornamental value (Glicenstein 2009; Hayden 2016). Jewel orchids are universally terrestrial plants. Most of them are photosynthetic but some Goodyerinae display myco-heterotrophic character. They usually produce white or yellowish flowers that emerge centrally from the plant’s stem, which is attached to a well-developed underground rhizome. Leaves are often, albeit not always, variegated in coloration. Many of the species are common in cultivation, including members of *Anoectochilus, Macodes* and *Dossinia* among others.

Taxonomic affiliation via DNA barcoding

L. discolor is very close morphologically to other Orchidaceae species, such as genera *Anoectochilus* and *Goodyera*. Therefore, molecular approach such as chloroplast genome comparison and DNA barcoding is applied for rapid and reliable taxonomic determination (**Chen et al.** 2019; Yu **et al.** 2019). The chloroplast genome of *L. discolor* is fully sequenced and it is characterized by the presence of a large signal-copy section of 82 922 base pairs and a small single-copy section of 26 572 base pairs (Yu **et al.** 2019). Such regions are very common in the genome of flowering plants and can be involved in a wide variety of functions (**Han et al.** 2014). They are often used to infer various phylogenetic relationships and to monitor key evolutionary events in the history of a taxonomic group or species in plants. In addition, the chloroplast genome of *L. discolor* includes another 132 genes, of which a total of 86 are protein-encoding. Thirty-eight genes are responsible for tRNA synthesis and eight genes for rRNA synthesis. Analysis and comparison of chloroplast genomes between individual plant populations show that the number and type of genes are highly conserved, but the length may vary. Accordingly, the genealogy of a given set of populations could be established depending on the length (Yu **et al.** 2019).

Plastid genes can be used as markers both for species identification and establishment of phylogenetic relationships through the marker methodological method DNA barcoding. The method relies on the amplification of short DNA fragments of genes whose genetic variation reflects the evolutionary history of plants. The set of such genes include *matK*(maturase K, tRNA-Lys*),psaB* (reaction centre protein of PSII), *rbcL* (ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit), *trnL, trnL-F (trnL-F* region composed of trnL intron and *trnL-F* intergenic spacer), and nuclear are: *ITS* (internal transcribed spacer 1 and 2 in rRNA) and*Xdh* (xanthine dehydrogenase gene; **Chen & Shiau** 2015; **Chen et al.** 2019). The goal of DNA barcoding is to create a shared source of DNA sequences that can be used to identify and determine taxonomic affiliation (**Hollingsworth et al.** 2011). Barcode of Life Data System (BOLD) is an information platform and repository that helps to acquire, store, and analyze DNA barcodes with open-public access (**Ratnasingham & Hebert** 2007).

16

To demonstrate the representation of available *in silico* data into individual phylogenetic trees for *L. discolor* we used the BOLD data base (Fig. 3). The DNA barcode sequences were pulled out from BOLD by search for the name of the plant species. The resulting list of sequenced *L. discolor* marker genes included information about two chloroplast and one nuclear DNA barcoding markers - *matK, rbcL* and *ITS,* respectively. The phylogenetic tree based on the *matK* marker (Fig. 3a) distinguished *Ludisia* as a sister genus of *Anoectochillus*, which is consistent with the ecological and morphological similarities between the two genera. The genus *Goodyera,* in turn, forms its own branch, and genus *Rhomboda* appears as a sister branch to *Ludisia.* In comparison with *rbcL* (Fig. 3b) and *ITS* (Fig. 3c), *matK* showed highest resolution. Combinations of the markers did not provide precise phylogenetic information (data not shown).

Photosynthetic adaptations in *L. discolor* and *Anoectochillus* sp. *L. discolor* and other jewel orchids are commonly characterized by an insufficient access to sunlight in their natural habitat. The uppermost layer of a forest usually receives between 1000-2500 ^mol m-2 s-1 photosynthetically active radiation, while only about 5 ^mol m-2 s-1 reach the understory (**Bolhar-Nordenkampf et al.** 2013). Due to that, species ecologically described as shade plants like *L. discolor, Anoectochillus* as well as many other *Goodyerinae* sp., have developed a set of photosynthetic adaptation traits such as distinct leaf structure, pigmentation and cytological features that help them to overcome these challenges. Sectioning coupled with microscopic analyses indicates that leaves of *L. discolor* and *Anoectochillus* sp. possess structural organization which is unique and unusual for monocotyledonous plant species (**Poobathy et al.** 2018). It is characterized by the presence of a dorsiventral arrangement of photosynthetic parenchyma differentiated into two layers, one of which is composed of spongy, while the other of palisade mesophyll (Fig. 4). Experiments with different types of light resulted in different fluorescent profiles of the two layers hinting at differences in pigmentation. Subsequent cytological observations as well as chromatographic analyses provide more information about the distribution and chemical nature of these pigments. The spongy mesophyll fluoresces in bright red when exposed to blue, green and ultraviolet wavelengths, which is considered as an indication for the presence of anthocyanins that are well known for their photoprotective role in plants. The vacuoles of both *L. discolor* and *Anoectochillus* sp. were discovered to possess large amounts of anthocyanins and fill the entire cellular space of the cells in the abaxial leaf layer. Chloroplasts are present irrespective of the anthocyanin content of the cells and demonstrate strong fluorescence under blue and ultraviolet light. The anthocyanins seem responsible only for the fluorescence under green light, since UV and blue light exposure results in a very dim glow. The cells that contain these pigments are spherical in shape and usually located right below the palisade layer. Furthermore, cyanidin was discovered as a major pigment in methanol extracts of both jewel

Anoectochilus chapaensis
Anoectochilus lytei
Anoectochilus elatus
Anoectochilus burmannicus
Anoectochilus albolineatus
Anoectochilus baotingensis
Anoectochilus calcareus
Anoectochilus hainanensis
Anoectochilus pingbianensis
Anoectochilus sikkimensis
Anoectochilus emeiensis
Anoectochilus longilobus
Anoectochilus nandanensis
Anoectochilus brevilabris
Anoectochilus malipoensis
Anoectochilus roxburghii
Anoectochilus zhejiangensis
Zeuxinella vietnamica
Odontochilus sp.
Anoectochilus lanceolatus
Pristiglottis montana
Pristiglottis sp.
Dossinia marmorata
Hetaeria affinis
Zeuxine affinis
Cheirostylis chinensis
Cheirostylis sp.

Fig. 3. Phylogenetic trees based on DNA barcoding markers. **A** - *matK* plastid gene. **B** - *rbcL* plastid gene. **C** - *ITS2* nuclear intergenic spacer. The sequences were pooled out from the BOLD database. The BOLD identification code and the one linked to the GenBank of NCBI are as follows: *matK* gene GBVR1719-13/AJ543911.1; *rbcL* gene GBVB3936-11/AJ542395.1; *ITS2* gene ITSAB3571-14/AJ539483.1. Each gene sequence is compared in the BLAST da
tabase to determine available proximity sequences. The obtained alignment of the *L. discolor* gene sequence with the 100 closest sequences available in the database (deposited by different experimental groups) was downloaded in FASTA format for subsequent phylogenetic analysis. To

determine the evolutionary interspecific relationships, evolutionary history is traced by ap plying the Neighbor-Joining method (S a i t o u & N e i 1987). A "bootstrap" phylogenetic tree was constructed (F el se n ste in 1985), in which the branches correspond to clusters of similar spe cies, and the percentage of similarity (based on a bootstrap test with 500 copies and similarity over 50%) is shown by number in the branches. Evolution distances were calculated using the Maximum Composite Likelihood method (T a m u r a e t a l . 2004) and are in units of the number of major field substitutions. The evolutionary analyzes were performed with the MEGA X program (K u m a r e t a l . 2018).

orchids but chromatographic analysis indicated that the mixture is complex and contains other related compounds such as procyanidin and pro-anthocyanidin, which serve as precursors in the biochemical processes leading to cyanidin.

Studies have also been conducted to establish the link between the photosyn-thetic processes in *Anoectochillus* and its metabolism, in particular its ability to produce various flavonoids that have potential antioxidant activity (**M a e t a l .** 2010). Light as a factor on the ability of orchids to inhibit various oxidative processes has been studied in *Phalaenopsis* (**A l i e t a l .** 2005). The data show that higher intensity increases superoxide dismutase levels in in vitro propagated individuals. *Anoectohillus*, on the other hand, it is a typical shade-loving plant, suitable for growing at photosynthetic photon flux (PPF) values in the range of 30 to 50 ^mol m-2 s-1. The saturation point is at about 60 ^mol m-2 s-1. Values of 90 ^mol m-2 s-1 caused stress, inhibited the natural course of photosynthesis through loss of activity in Photosystem II (PSII) and led to a decrease in the concentration of flavonoids. There was also a significant decrease in the concentration of chlorophyll, while its highest levels are at PPF values of 10 ^mol m-2 s-1. In the range of increase from 10 to 60 ^mol m-2 s-1 there was an increase in the ratio between chlorophyll *a* and chlorophyll *b* and a decrease in the size of the light-gathering complex of PSII. This was accompanied by an increase in the concentration of flavonoids which can be explained by the fact that a greater light intensity increases oxidative stress, and the plant changes its biochemical parameters

Fig. 4. Leaf lamina cross-section of *L discolor*. Photosynthetic pa renchyma differentiated into two layers: palisade (P) and spongy (S) mesophyll. Scale bar 50 |jm. (section is made by V.A.H.)

to prevent the negative impact. However, very high levels of illumination led to a sudden drop in flavonoid levels. Research sug-gests that light could be successfully used as a factor by which the biosynthesis of various biologically active substances can be purposefully regulated.

Mycorrhizal interactions

Mycorrhizal associations represent a widespread form of symbiotic interaction in plants and recent research reveals that they can be formed by nearly 90% of all land species (Soudziloveskaia et al. 2020). For orchids, these associations are not only universal but quintessential in the early stages since the lack of endosperm in the miniature seed requires the presence of a fungal partner to achieve successful protocorm development (Rasmussen 2002). Some orchids, ecologically belonging to the group of myco-heterotrophic plants, spend their entire life in an obligatory symbiosis with fungi. Such species are described within the jewel orchid group, with the genus *Chamaegastrodia* as a major example. Analysis of *L. discolor* specimens collected from various locations in Thailand resulted in the isolation of *Ceratorhiza goodyerea-repentis* (Athipunyakom et al. 2004). As the name implies, this species is associated with a temperate relative of *L. discolor,* namely *Goodyera repens.* *Ceratorhiza goodyerea-repentis* was also isolated from another species of *Goodyera,* namely *G. procera,* which also demonstrates the presence of *C. cerealis.* However, genetic analyses of fungi from the related *G. pubescens,* which grows in North America, identifies species from the genus *Tulasnella* as the only symbiont that is present in this orchid (McCormick et al. 2004). Studies on the genetic identification of fungal isolates from *L. discolor* and different *Anoectochillus* species collected from China have provided intriguing data regarding their effect on *A. roxburghii* (Ye et al. 2020). A total of 277 different strains have been successfully isolated that belonged to a diverse set of genera such as *Gliomastix*, *Bjerkandera*, *Auricularia*, *Helminthosporium, Colletotrichum, Acremonium, Bionectria, Fusarium, Hypoxylon,* Xylariaceae, *Diaporthe, Phomopsis* and *Chaetomium.* A growth promoting and biomass increasing effect was observed for those isolates when co-cultivated with *Arabidopsis thaliana.* Two fungal strains, which were subsequently identified as *Chaetomium globosum* and *Colletotrichum gloeosporioides,* were also revealed to have a prominent beneficial effect on *A. roxburghii.* They appear to promote the accumulation of various chemical components such as flavonoids, kinsenosides and polysaccharides, many of which represent an interesting object for research given their potential medical application. Histochemical analysis shows that the

two species of fungi are endophytic in nature and colonize the intercellular gap of xylem parenchyma cells in roots without inhibiting the development of the host. Isolation of fungi such as *Ceratorhiza* and *Tulasnella* is not surprising since they belong to the group of the so called *Rhizoctonia* complex, a taxonomically loose set of fungi which are commonly associated with orchids and considered to be their major symbionts (Yang & Li 2012). The species isolated from the Chinese-collected plants,

however, unfold an important object of interest. Many of these fungi such as *Acremonium, Hypoxylon, Diaporthe* and many others are ascomycetes, which is considered very unusual since the only orchid known to form symbiosis with members of this phylum is the unrelated *Epipactis,* a genus associated with species in the genus *Tuber* (Selosse et al. 2004). More research on this topic would be necessary to uncover the enigmatic biological nature of these potentially novel interactions in the orchid family and give more details about its effect on the development of *Ludisia* and *Anoectochillus* species in their native environment.

Traditional medicine

Orchids (including *L. discolor)* are commercially used in Chinese and South Asian traditional medicine systems (Teoch 2016; Leon & Lin 2017). Numerous patents were generated using *L. discolor* as few of them are shown in Table 1. In Thailand's primary health care practices, *L. discolor* is listed among the herbs used in the treatment of venomous snakebites (Wongkongkathep et al. 2017).

Table 1. *L. discolor* patents.

Patent	Description
CN105963562A, 2016	of QI of the lung and kidney. In this drug composition is made by *L. discolor,* herba; *Juglans regia,* smen; *Davallia solida,* rhi zome; *Oroxylum indicum,* semen; *Trichosanthes kirilowii,* pericarpium.
CN103705742B, 2016	
CN105053354A, 2015	Patent about the kyllinga monocephala rottb lung-dispersing and cough-re lieving tea is prepared by nine herbs, one of them is *L. discolor,* and has the effects of ventilating lung, relieving cough, clearing heat, moistening lung and eliminating phlegm when being taken for a long time.
CN104940625A, 2015 The traditional Chinese medicine composition with *L. discolor*, all herbs are combined to treat muscular atrophy side effects caused by creotoxin and also play an auxiliary treatment role on cerebral trauma spasm.	Traditional Chinese Medicine composition for treating chronic obstructive lung disease. Herbal composition from 16 ingredients, include *L. discolor,* and used as decoction with water.
The emophysematous Chinese medicine composition for the treatment of deficiency type	

According to the Thai ancient textbook, *L. discolor* (rhizome) is an ingredient of a recipe used for relieving the symptoms of hyperacidity syndrome (Sireeratawong et al. 2013). The whole plant is utilized in Traditional Chinese Medicine and it may be collected throughout the year and used fresh or dry (sun-dried). The herb is known as sweet and astringent, efficient against lung complaints, regulator of body fluids,

blood purifier and anti-inflammatory and blood clotting agent. It is also used to treat haemoptysis caused by pulmonary tuberculosis, neurasthenia, anorexia and as a cough relive and decoction (dry or fresh herb). The herb can be chewed fresh or used to produce beverages (**Li** 1998; **Teoch** 2016). An ethnobotanical study on medicinal plants in Hainan Island, China showed that local people use whole plants of *L. discolor* for external treatments of injuries (**Zheng & Xing** 2009). *Anoectochilus roxburghii* is officially designated as the only source of local medicinal herb Jinxianlian (Wu **et al.** 2020). Some clinical applications of *Goodyera* and *Ludisia* species are similar to those of *A. roxburghii,* such as their utilization in the treatments of tuberculosis hemoptysis, rheumatism, rheumatoid arthritis and after snake bite (**Ai** 2013).

Phytochemistry and biological activities

Three genera of jewel orchids, namely *Ludisia*, *Anoectochillus* and *Goodyera*, have been studied for their phytochemical content and its potential biological activity (Du **et al.** 2008; Wu **et al.** 2020). Key compounds extracted from these plants are kinsenoside, goodyeroside A and goodyeroside B. Their structures have been confirmed through mass spectroscopic analyses (Table 2 ; **Rehman et al.** 2016). These molecules that represent isomers of each other belong to the class of lactone glycosides - they have been extensively isolated from jewel orchids, with kinsenoside and goodyeroside A also extracted from the unrelated *Crocus sativus* (**Righi et al.** 2015).

Table 2. Phytochemical content.

Metabolite	Chemical formula	PubChem Identifier https://pubchem.ncbi.nlm.nih.gov/	References
Gastrodin	$C_{13}H_{18}O_7$	115067	Pang et al. 2018
Goodyerin	$C_{36}H_{40}O_{19}$	102460727	Du et al. 2002
Goodyeroside	$C_{10}H_{18}O_9$	10445498	Zhang et al. 2005
Kinsenoside	$C_{10}H_{16}O_8$	10422896	Zhang et al. 2005

The lactone glycosides derivatives are characterized by the presence of a glucopyranosyl moiety bonded via a glycosidic bond to a butyrolactone ring. There are a total of 6 chiral centers, one of them located in a ring giving the potential for a variety of other isomers (**Zhang et al.** 2005). There is evidence that these phytochemicals demonstrate hepatoprotective, antihyperglycemic, antioxidant, anti-inflammatory and antihyperliposis activities (Table 3). Early experiments

Table 3. Biological activities.

Biological activity	Extraction notes	Reference
Anti-diabetic	Pre-extracted kinsenoside	Zhang et al. 2007
Anti-hyperliposis	Reverse-phase silica fel column chromatography without methanol	Du et al. 2001
Antioxidant	Ethanol treatment, chromatography	Liu et al. 2014
Hepatoprotective	Water extraction, filtration, concentrating at reduced pressure	extraction at room temperature Lin et al. 1993 Du et al. 2002
Sedative and anti-convulsant	Methanol	
Vascular protective	Water extraction at 90°C	Liu et al. 2017

investigated the effect of *A. formosanus* extracts on tissues from rats with acute hepatitis that was induced through carbon tetrachloride and acetaminophen treatment (**Lin et al.** 1993). Analysis of the samples shows that the induced cell damage has been inhibited in primary cultured hepatocytes isolated from the animals. Later research discovered that the active constituents of these extracts are kinsenoside and its related compounds (Wu **et al.** 2007). Its potential antidiabetic activity has been evaluated through in vivo studies, which indicated that orally administering different doses of kinsenoside to rats results in treated groups having a higher number of intact P cells in the islets of Langerhans (**Zhang et al.** 2007). The anti-hyperliposis activity of the jewel orchid compounds was demonstrated on rat models where the animals were fed with a high fat diet. Animals that were given kinsenoside showed a reduction in weight, however goodyeroside A failed to exert such effect (Du **et al.** 2001). The latter compound is considered to have a generally lower biological activity according to the available literature stressing on the absence of anti-diabetic and anti-autoimmune effect. On the other hand. goodyeroside B appears to be poorly studied and its potential activity needs to be further investigated. By contrast to its related compounds, there exist an increased number of preclinical studies regarding the metabolic stability of kinsenoside. The studies employ liver microsomes, subcellular structures representing vesicles containing a set of common liver enzymes that often damage various xenobiotics and impede the potential exploitation of novel drugs with various biological activities. Recent research involving HPLC allowed to determine the quantitative content of lactone glycosides in the three major members of the terrestrial orchid group and showed variation depending on both taxonomy and growth conditions. *Anoectochillus* species differ drastically in their kinsenoside content with values ranging from 3.38 to 229.17 mg/g (Wu **et al.** 2020). Interestingly, tissue-cultured samples of *A. roxburghii* and *A. formosanus* display higher yields of kinsenoside compared to specimens collected from the wild. It implies that artificially grown plants are better candidates for mass extraction compared to orchids in their native

habitat. The presence of kinsenoside has also been confirmed in both *Ludisia* and

Goodyera, with the highest concentration registered for *G. schlechtendaliana.* Both genera are rich in goodyeroside A with a concentration from 79.6 up to 150.61 mg/g, while the values for kinsenoside are lower compared to what has been determined for *Anoectochillus*. Besides being rich in lactone glycosides, *Anoectochillus,*
Ludisia and *Goodyera* contain an abundant variety of other biological compounds. Another type of glycoside, goodyerin, which belongs to the flavonol class has been demonstrated to have sedative properties (Du et al. 2002). Tests with phenol and sulfuric acid show high polysaccharide content in most sampled species of jewel orchids, apart from *G. biflora* and *A. burmannicus*, where the concentration appears relatively low. Such polysaccharides have been studied for their biological activity as well and there is evidence for hepatoprotective, antidiabetic and vascular protective properties similarly to data for kinsenoside. Other phytochemicals are various flavonoids, gastrodin, isorhamnetin, quercetin, narcissin and kempherol that were isolated from these plants, too (Wu et al. 2020). Many of them are reported to have antioxidant, anti-cancer or anti-autoimmune activities, however, further in vivo tests are necessary for a better evaluation of any potential pharmacological effect.

Micropropagation

In contrast to many other members of its family, *L. discolor* can be readily propagated via rooting of stem cuttings or separation of individuals from a larger tuft (Jackson 2005). The plant does not require any special conditions and usually demonstrates vigorous development. The combination of these factors make *L. discolor* an excellent model organism for the orchid family, which sparks interest in its application for plant research in the lab. Skills in its micropropagation are vital for this purpose since they allow the maintenance of many individuals in a sterile environment and provide an important tool for analyses of its pharmacological properties. Sterile conditions keeping the explants free from contamination has a key role for the success of this process. Sterilization procedures involving a combination of ethanol rinses plus various concentrations of sodium hypochlorite or mercury chloride have been tested (Poobathyetal.
2019). The analyses show that mercury chloride at concentration 0.4 w/v is the most suitable choice for this purpose. Various growth media have been tested as well, including half strength Murashige and Skoog (MS), modified Knudsen C and MS basal medium combined with tryptone and gelrite (Table 4). Half strength MS with added naphthyl acetic acid (NAA), thidiazuron and active charcoal gives optimal development of the explants, since *L. discolor* appears to be sensitive to high concentration of nutrients. Acclimatization of individuals can be achieved with coconut coir, coconut husk and peatmoss (Poobathyetal. 2019). The formation of adventitious bud formation in *Ludisia* has been studied as well and the effects of 6-benzylaminopurine (6-BA), NAA, Cu2+ and Ag2+ were tested.

Table 4. Media variants used for the micropropagaion of *L. discolor.* Variant

Composition	Reference
T1 Half-strength Murashige and Skoog (Murashige and Skoog, 1962) basal medium, 0.2% (w/v) activated char coal, 8% (w/v) Mas banana cultivar homogenate, 3% (w/v) sucrose, 3.5 g L-1 Gelrite, 1.0 mg L-1 1-naphthale neacetic acid (NAA), and 0.1 mg L-1 thidiazuron (TDZ)	NAA, CuSO4, AgCl, sucrose, inositol Poobathyetal. 2019 Liuetal. 2021
T2 Knudson C	
T3 Modified Knudson C	
T4 MS basal medium, 3 g L-1 tryptone, 30 g L-1 sucrose, 2.75 g L-1 Gelrite (pH 5.2)	
Adventitious bud induction MS basal, 6-BA,	
Seed germination A Half strength Murashige and Skoog	Shiauetal. 2005
Seed germination B Half strength Hyponex № 1, 2.00 mg/L 6-BA, 0.6 mg/L NAA, coconut milk	Hongyangetal. 2016

The experiment indicates that best results are produced with a combination of 1.0 mg/L 6-BA, 0.75 mg/L NAA plus 0.25 mg/L CuSO4 or 6.4 mg/L AgCl. The phytoeffectors 6-BA, Ag2+ and Cu2+ show an inhibitory effect as the concentration increases, however, high concentration of NAA is well tolerated and leads to a better regeneration rate (**Liuetal.** 2021).

Successful asymbiotic germination of seeds from *L. discolor* is also reported in at least two studies (**Shiauetal.** 2005; **Hongyangetal.** 2016). Both use immature capsules produced after hand pollination. This avoids the sterilization of the seeds since the interior of the capsule is considered free of contaminants and only its surface is subjected to treatments with both bleach and mercury chloride resulting in effective sterilization. Similarly to other orchid species, immature seeds demonstrate a higher germination rate. However, more tests would be helpful to determine the optimal sowing medium since both studies contradict each other with respect to what is considered the best choice for *L. discolor*. In one of the studies, half strength MS leads to the most desired results (**Shiauetal.** 2005), while in the other study (**Hongyanget al.** 2016) Hyponex is reported to produce the highest germination rate with half-strength MS appearing to be inappropriate. Nevertheless, the addition of regulatory substances such as NAA, TDZ or 6-BA has an unambiguously simulative effect on the seedling's development. About four to five months are usually required for seedlings to reach an early stage of maturity

after which they can be transferred to greenhouse conditions.

CONCLUSION

Ludisia discolor could serve as a model organism for studying fundamental features in the biology of orchids - plants that are usually more demanding in terms of cultivation and reproduction conditions. For this reason, the exploration of its biochemical, physiological, reproductive, and other characteristics is essential to reveal the great potential that *L. discolor* can offer. DNA marker techniques like DNA barcoding open new horizons for taxonomic characterization of orchids both in terms of better understanding the genetic variability and species dynamics at ecology level but also to elucidate the complex network of mycorrhizal associations within this plant family.

Ludisia discolor is also rich in biologically active substances, including some key compounds such as kinsenoside, gudieroside and gudierin, which are of pharmacological interest. The study of orchids and the development of successful techniques for its reproduction can open new opportunities for mass production of these compounds and their potential application in medicine. In summary, *L. discolor* could be used for decorative purposes, as well to produce economically important products, which increases the interest of more detailed studies.

CONFLICT OF INTERESTS

The authors declare that there is no conflict of interests regarding the publication of this article.

ACKNOWLEDGEMENTS

This work was supported by grant №80-10-69/11.05.2022, of the fund for scientific investigations of Sofia University “St. Kliment Ohridski”. DNA barcoding analyses were performed in the frame of the project BULCode No. Д01-271/02.10.2020, National Program “European Scientific Networks” funded by the Ministry of Education and Science of Bulgaria.

AUTHORS CONTRIBUTION

All authors contributed equally to the design, discussion and writing of the manuscript.

References

Ai T.-M. 2013. Medicinal Flora of China. Volume 12. Peking University Medical Press, Beijing, China, 797 pp.

Ali M. B., **Hahn** E. J. & **Paek** K. Y 2005. Effects of light intensities on antioxidant enzymes and malondialdehyde content during short-term acclimatization on micropropagated *Phalaenopsis* plantlet. - Environ. Exp. Bot. 54 (2): 109-120.

Athipunyakom P., **Manoch** L. & **Piluek** C. 2004. Isolation and identification of mycorrhizal fungi from eleven terrestrial orchids. - Agric. Nat. Resour. 38 (2): 216-228.

Averyanov L. V., Loc P. K., **Hiep** N. T. & **Harder** D. K. 2003. Phytogeographic review of Vietnam and adjacent areas of Eastern Indochina. - Komarovia 3: 1-83. **Barcode of Life Data System.** https://www.boldsystems.org/ (Last accessed on 31.05.2022).

Chase M. W., **Cameron** K. M., **Freudenstein** J. V., **Pridgeon** A. M., **Salazar** G., **van den Berg** C. & **Schuiteman** A. 2015. An updated classification of Orchidaceae. - Bot. J. Linn. Soc. 177 (2): 151-174.

Chen S. P., **Tian** H. Z., **Guan** Q. X., **Zhai** J. W., **Zhang** G. Q., **Chen** L. J., Liu Z. J., **Lan** S. R. & **Li** M. H. 2019. Molecular systematics of Goodyerinae (Cranichideae, Orchidoideae, Orchidaceae) based on multiple nuclear and plastid regions. - Mol. Phylogenet. Evol. 139: 106542.

Chen J.-R. & **Shiau** Y.-J. 2015. Application of internal transcribed spacers and maturase K markers for identifying *Anoectochilus*, *Ludisia*, and *Ludochilus.* - Biol. Plant. 59 (3): 485-490.

Du X.M., **Irino** N., **Furusho** N., **Hayashi** J. & **Shoyama** Y. 2008. Pharmacologically active compounds in the *Anoectochilus* and *Goodyera* species. - J. Nat. Med. 62 (2): 132-148.

Du X. M., **Sun** N. Y., **Takizawa** N., Guo Y T. & **Shoyama** Y. 2002. Sedative and anticonvulsant activities of goodyerin, a flavonol glycoside from *Goodyera schlechtendaliana.* - Phytother Res. 16 (3): 261-263.

Du X., **Sun** N., **Tamura** T., **Mohri** A., **Sugiura** M., **Yoshizawa** T., **Irino** N., **Hayashi** J. & **Shoyama** Y. 2001. Higher yielding isolation of kinsenoside in *Anoectochilus* and its anti-hyperliposis effect. - Biol. Pharm. Bull. 24 (1): 65-69.

Glicenstein L. 2009. Jewel and painted-leaf orchids: I. *Ludisia* and *Anoectochilus.* - Orchids 78 (11): 664-669.

Bolhar-Nordenkampf H. R. & **Draxler** G. 2013. Functional leaf anatomy. In: **Hall** D. O., **Scurlock** J. M. O., **Bolhar-Nordenkampf** H. R., **Leegood** R. C. & **Long** S. P. (Eds.), Photosynthesis and production in a changing environment: a field and laboratory manual. Springer Science & Business Media, Dordrecht, 326 pp.

Han F., **Peng** Y., Xu L. & **Xiao** P. 2014. Identification, characterization, and utilization of single copy genes in 29 angiosperm genomes. - BMC Genom. 15 (1): 1-9.

Hayden W. J. 2016. Jewels of the Orchidaceae. - Sempervirens Quarterly (Fall 2016), 6-7.

Hollingsworth P. M., **Graham** S. W. & **Little** D. P. 2011. Choosing and using a plant DNA barcode. - PloS ONE, 6 (5): e19254. doi:10.1371/journal. pone.0019254

Hongyang L. I., **Yang** L. I. U., **Guanming** C. H. E. N. & **Zhijuan** Y. A. N. G. 2016. Aseptic Seedling and Rapid Propagation of *Ludisia discolor*. - Agric. Sci. Technol. 17 (11): 2473-2476, 2482.

Jackson A. M. 2005. Determination of optimal rooting conditions for stem cuttings of *Ludisia discolor*. Master Thesis, Mississippi State University, 51 pp. **Kew Science**, 2020. Plants of the world online. http://powo.science.kew.org/taxon/ urn:lsid:ipni.org:names:641773-1 (Last accessed on 31.05.2022). **Kumar** S., **Stecher** G., **Li** M., **Knyaz** C. & **Tamura** K. 2018. MEGA X: Molecular Evolutionary Genetics Analysis across Computing Platforms. - Mol. Biol. Evol. 35 (6): 1547-1549.

Leon C. & **Lin** Y.-L. 2017. Chinese medicinal plants, herbal drugs and substitutes: an identification guide. Kew Publishing, Royal Botanic Gardens, Kew, 850 pp. **Li** N.H. (Ed.) 1998. Chinese medicinal herbs of Hong Kong, vol. 2. Hong Kong Chinese Medicinla Research Institute, Hong Kong, 198 pp.

Lin J.M., **Lin** C.C., **Chiu** H.F., **Yang** J.J. & **Lee**, S.G. 1993. Evaluation of the anti-inflammatory and liver-protective effects of *Anoectochilus formosanus*, *Ganoderma lucidum* and *Gynostemma pentaphyllum* in rats. - Am. J. Chinese Med. 21 (01): 59-69.

Liu Y., **Li** X., **Chen** J., **Xue** Y. & **Zhu** Y. 2021. Effects of different factors on adventitious bud induction from stem explants of *Ludisia discolor*. - In: **Duan** L. & **Abdullah** A.Z. (Eds.), E3S Web of Conferences 245, 5th International Conference on Advances in Energy, Environment and Chemical Science, 26-28 February 2021, Shanghai, China, p. 03024.

Ma Z., **Li** S., **Zhang** M., **Jiang** S. & **Xiao** Y 2010. Light intensity affects growth, photosynthetic capability, and total flavonoid accumulation of *Anoectochilus plants*. - HortScience 45 (6): 863-867.

McCormick M. K., **Whigham**, D. F. & **O'neill** J. 2004. Mycorrhizal diversity in photosynthetic terrestrial orchids. - New Phytol. 163 (2): 425-438. **Poobathy** R., **Zakaria** R., **Murugaiyah** V. & **Subramaniam** S. 2018.Autofluorescence study and selected cyanidin quantification in the Jewel orchids *Anoectochilus* sp. and *Ludisia discolor*. - PLoS ONE 13 (4): e0195642.

Poobathy R., **Zakaria** R., **Murugaiyah** V. & **Subramaniam** S. 2019. Surface sterilization and micropropagation of *Ludisia discolor*. - Biocatal. Agric. Biotechnol. 22: 101380.

Ratnasingham S. & **Hebert** P. D. 2007. BOLD: The Barcode of Life Data System (http://www. barcodinglife. org). - Mol. Ecol. Notes 7 (3): 355-364. **Rasmussen** H. N. 2002. Recent developments in the study of orchid mycorrhiza. -

Plant soil 244 (1): 149-163.

Rehman S. U., **Kim** I. S., **Choi** M. S., Luo Z., **Yao** G., **Xue** Y., **Zhang** Y. & Yoo H. H. 2016. Development of a hydrophilic interaction liquid chromatography tandem mass spectrometric method for the determination of kinsenoside, an antihyperlipidemic candidate, in rat plasma and its application to pharmacokinetic studies. - J. Pharm. Biomed. Anal. 120: 19-24.

Righi V., **Parenti** F., **Tugnoli** V., **Schenetti** L. & Mucci A. 2015. *Crocus sativus* petals: waste or valuable resource? The answer of high-resolution and High resolution magic angle spinning nuclear magnetic resonance. - J. Agric. Food Chem. 63 (38): 8439-8444.

Saitou N. & **Nei** M. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. - Mol. Biol. Evol. 4 (4): 406-425. **Selosse** M. A., **Faccio** A., **Scappaticci** G. & **Bonfante** P. 2004. Chlorophyllous and achlorophyllous specimens of *Epipactis microphylla* (Neottieae, Orchidaceae) are associated with ectomycorrhizal septomycetes, including truffles. - Microb. Ecol. 47 (4): 416-426. **Shiau** Y. J., **Nalawade** S. M., **Hsai** C. N. & **Tsay** H. S. 2005. Propagation of*Haemaria discolor* via in vitro seed germination. - Biol. Plant. 49 (3): 341-346. **Sinovas** P., **Price** B., **King** E., **Hinsley** A. & **Pavitt** A. 2017. Wildlife trade in the Amazon countries: an analysis of trade in CITES listed species. Technical report prepared for the Amazon Regional Program (BMZ/DGIS/GIZ). UN Environment - World Conservation Monitoring Centre, Cambridge, UK, 113 pp. **Sireeratawong** S., **Nanna** U., **Vannasiri** S., **Chiruntanat** N., **Srithiwong** S., **Subcharoen** P., **Termwiset** P., **Tungsukruthai** P., **Chuthaputti** A. & **Jaijoy** K. 2013. Acute and subchronic toxicity study of Tud-Rak-Ka-Sai-Puu recipe in rats. - Afr. J. Tradit. Complement. Altern. Med. 10 (1): 142-148. **Soudzilovskaia** N. A., **Vaessen** S., **Barcelo** M., **He** J., **Rahimlou** S., **Abarenkov** K., **Brundrett** M. C., **Gomes** S. I., **Merckx** V. & **Tedersoo** L. 2020. FungalRoot: global online database of plant mycorrhizal associations. - New Phytol. 227 (3): 955-966.

Tamura K., **Nei** M. & **Kumar** S. 2004. Prospects for inferring very large phylogenies by using the neighbor-joining method. - Proc. Natl. Acad. Sci. U.S.A. 101 (30): 11030-11035.

Teoh E. S. 2016. Medicinal orchids of Asia. Springer Nature Switzerland AG, 770 pp.

Wongkongkathep S., **Prasertsiripong** N., **Stienrut** P., **Chantraket** R. & Sonksakda M. (Eds.). 2017. Thai traditional and alternative health profile: Thai traditional medicine, Indigenous medicine and alternative medicine 2014-2016. Technical Services Bureau, Department for Development of Thai Traditional and Alternative Medicine, Ministry of Public Health, 357 pp.

Wu J. B., **Lin** W. L., **Hsieh** C. C., Ho H. Y., **Tsay** H. S. & **Lin** W. C. 2007. The hepatoprotective activity of kinsenoside from *Anoectochilus formosanus.* -