CAHIERS DE
LA SOCIÉTÉ FRANÇAISE D'ORCHIDOPHILIE

N°9 – 2018

ORCHIDÉES PARIS 2018

18th European Orchid Council Conference and Exhibition Proceedings

What future for orchids?

Proceedings of the 18th European Orchid Council Conference and Exhibition

Scientific conference

What future for orchids?

24-25 March 2018
Paris Event Center, Paris

On behalf of *L'orchidée en France*

Conference organizing committee:
Richard Bateman, Alain Benoît, Pascale Besse, Yves Henry, Jana Jersákowá, Ray Ong, Daniel Prat, Marc-Andre Selosse, Tariq Stevart

Cover photography from Philippe Lemettais

Proceeding edition: Daniel Prat

Cahiers de la Société Française d'Orchidophilie, N° 9, Proceedings of the 18th European Orchid Council Conference and Exhibition – Scientific conference: What future for orchids?

ISSN 2648-2304 en ligne

Proceedings of the 18th European Orchid Council Conference and Exhibition – Scientific conference: What future for orchids? SFO, Paris, 2018, 166 p.

Société Française d'Orchidophilie
17 Quai de la Seine, 75019 Paris

Foreword

The first European Orchid Council Conference and Exposition (EOCCE) was organized in 1967 in Vienna. The second conference followed 2 years later in 1969, together with the Floralies in Vincennes, Paris. 19 years later, in 1988 the EOCCE was again in Paris, the conference program was in a building at the Trocadero, the orchid exhibition was in a tent on the Champs de Mars, both localities with the perfect view to the most famous landmark of Paris, the Eiffel-tower. I still remember the storm during one afternoon, strong enough to force the responsible of the organization committee to shut down the exhibition for some hours. And now in 2018 we saw the 3rd EOCCE again in Paris, not in the heart of the town, but not too far away.

The organization committe unified 4 different French societies, the Société Française d'Orchidophilie (SFO), the Société Nationale d'Horticulture de France (SNHF), the Fédération Française des Amateurs d'Orchidées (FFAO) and France Orchidées.

The scientific program was held under the title "What future for Orchids". Part of the conference was also an editors meeting, bringing together the editors of the leading orchid periodicals of Europe and resulting finally in an article-exchange project between different journals. Other meetings were held about orchid judging and orchid culture. A large poster exhibition showed results of the ongoing research in connection with this fascinating plant family. Unfortunately we don't have proceedings of all EOCCE's, it is very positive that we will have them from the one in Paris

Are there perfect shows and conferences existing? I doubt it, I have seen over the years a huge number of shows and conferences all over the world. There was always a very dedicated team of a few persons behind the event, trying very hard to get everything perfectly organized and planned. We should never forget that everything was done by non-professionals in their free time. There are always things which cannot be planned at all, and it ends always with a certain amount of improvisation.

The EOCCE in Paris was well organized and the orchid exhibition was impressive. On behalf of the European Orchid Council I would like to express the sincere thanks to the organization committee LOF.

Rudolf Jenny
Secretary General of the European Orchid Council

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Conservation status of a species indicates how likely the species will go extinct in the future. Many factors are considered to determine the status: population size with its overall increase or decrease over time, reproduction success, and known threats. Various systems of assessment of conservation status are used at international, multi-country, national and local levels. The most well-known is certainly IUCN red listing.

Cypripedium calceolus L. – lady's-slipper orchid, is largely recognized in Europe as an emblem for nature conservation. The species distribution is scattered widely in Eurasia but throughout most of the range it is highly fragmented. In the IUCN red list the species is in the category LC-least concern as "neither the geographic range of the species nor the size of the populations fall within the thresholds for any of the threatened categories and the existing threats for the species and habitats are unlikely to cause the populations to decline quickly in the near future". The regional European assessment categorizes the species as NT (near threat) and EU conservation status in all biogeographical regions is unfavourable-inadequate. The species is listed as threatened in most national red lists of Europe and has become extinct in some. *C. calceolus* is threatened by habitat destruction that includes agriculture intensification, inappropriate forest management such as clear cutting, also replacement of natural forests with spruce plantations where decalcification processes spoil the soil for the orchid, and collection. Also, overgrazing affects the species, while the abandonment of traditional grazing activities leads to natural succession where the over-growing bush layer outcompetes the orchid.

How much should we really bother about the fate of this species and what can we do? There are certain traits that may be crucial for the long-run persistence of populations:

- small population size - low number of genets (even a population of 1000 shoots may have only 100 genets or clones);
- clones are long-lived only in favourable conditions and are not eternal;
- deceiving pollination system enables only low fruit set – usually less than 25 per cent of flowers set fruit;
- fruits contain many seeds but about half of them are non-viable without embryos, even in Estonian populations with high genetic diversity;
- seeds germinate only with the help of *Tulasnella* fungi and seedlings are rare and the mortality of them high as in many species;
- the populations are not well connected due to lack of suitable habitats.

During the last decades the species has been successfully propagated asymbiotically and the seedlings planted to natural populations leading to successful restoration. These techniques together with assurance of high genetic diversity of progeny should be more actively employed and many parks and woods on calcareous soils could inhabit lady's-slippers to enlarge the number and size of populations on larger areas giving better connectivity and possibilities for crossing.

Species distribution models and their application in orchid biodiversity research

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Species distribution models (SDMs) are numerical tools that combine observations of species occurrence or abundance with environmental estimates. They are used to gain ecological and evolutionary insights and to predict distributions across landscapes, sometimes requiring extrapolation in space and time. SDMs are now widely used across terrestrial, freshwater, and marine realms. Differences in methods between disciplines reflect both differences in species mobility and in "established use." Model realism and robustness is influenced by selection of relevant predictors (climatic variables, geological substrate, slope and orientation, etc.), by modeling method (usually MaxEnt program, but other models can also be used), spatial scale considered (region, country, continent), and by the extent of extrapolation (i.e., how far we extrapolate from the sites where data were collected).

Biologically speaking, SDMs enable to predict occurrence of the species in question based on a set of GPS coordinates of the known sites and a set of biotic and abiotic characteristics of these sites. The output of these models is a map of potential distribution of the species, where the likelihood of its occurrence (a number between zero and one) is depicted in the same way as, e.g., altitude in classical maps – usually by different colors.

The species distribution models are especially useful in regions, which were not yet fully explored, as they enable to pinpoint sub-regions of potential occurrence of the species studied. Based on such results, areas with special protection may be declared. We will show some examples of such maps using data on several orchid species.

Tree removal as a management strategy for the lady's slipper orchid

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Abstract – The lady's slipper orchid (*Cypripedium calceolus*) grows as an understory herb in boreal herb-rich forests, where the dominance of spruce often decreases light and nutrient availability. To study selective tree removal as a management method in northern Finland, we used long-term demographic data from ten unharvested control sites and ten harvest sites which were divided into three treatments with differing tree harvest intensity: (1) dense spruce forests, where half of the total tree basal area (TBA) was cut, (2) sparse spruce forests, where one-fourth of the spruce TBA was cut and (3) sparse broadleaf forests, where one-fourth of the total TBA was cut. Orchid flowering and fruiting probabilities were higher at the harvested spruce forests sites compared to control sites, while harvesting increased survival and ramet density at the moderately harvested broadleaf forest sites. The effects on flowering and fruiting probabilities and survival disappeared quickly, whereas ramet density responded only with a lag of several years. Tree removal had no effect on dormancy or seedling density. Our results demonstrate that for the lady's slipper orchid, a flagship species for nature conservation, the selective tree harvest might be a suitable management method that increases fruit production and the population size at the ramet level.

This is an abridged, author-produced version of an article accepted for publication in Forest Ecology and Management following peer review. The version of record Hurskainen, S., Jäkäläniemi, A., Ramula, S., & Tuomi, J. (2017). Tree removal as a management strategy for the lady's slipper orchid, a flagship species for herb-rich forest conservation. *Forest Ecology and Management, 406*: 12-18 *is available online at:* http://dx.doi.org/10.1016/j.foreco.2017.09.056

INTRODUCTION

Understory light conditions and associated changes in e.g. temperature and moisture, are considered to be the major limiting components of forest understory cover and species richness in temperate and boreal forests (Barbier *et al.*, 2008). In addition to light, canopy closure affects many abiotic and biotic factors of the forest understory, which in turn can modify, both directly and indirectly, the growth, survival, and reproduction of understory plants (Figure. 1). In Finland, spruce-dominated old-growth forest represents a natural part of the succession cycle of herb-rich forests. *P. abies* offers shelter and substrate for certain birds and decomposing fungi (Alanen *et al.*, 1995), but also effectively shades the understory and produces acidic litter that decomposes slowly (Alanen *et al.*, 1995; Zhang *et al.*, 2008), and the subsequent resource limitation can be detrimental for herbaceous understory species.

The lady's slipper orchid (*Cypripedium calceolus* L.) is a rare understory herb which prefers half-shaded lime-rich habitats (Rankou and Bilz, 2014). Because of its large, showy flowers, the lady's slipper orchid is an ideal flagship species to attract public interest in conservation. Moreover, due to its stable population dynamics, occurrence on rare habitat types, and tendency to co-occur with several other rare orchids, herbs, and mosses, it has been suggested that the lady's slipper orchid could be used as an umbrella species (Bjørndalen, 2015; Laitinen, 2006; Nicolè *et al.*, 2005). In other words, the decline of the lady's slipper orchid indicates the degradation of the habitat, and therefore, an improvement in conditions for this orchid might ensure the survival of other species that are dependent on similar habitats (Simberloff, 1998).

Previous studies have shown that the viability of lady's slipper orchid populations decreases with increasing canopy closure in boreal and nemoral forests (Brzosko, 2002;

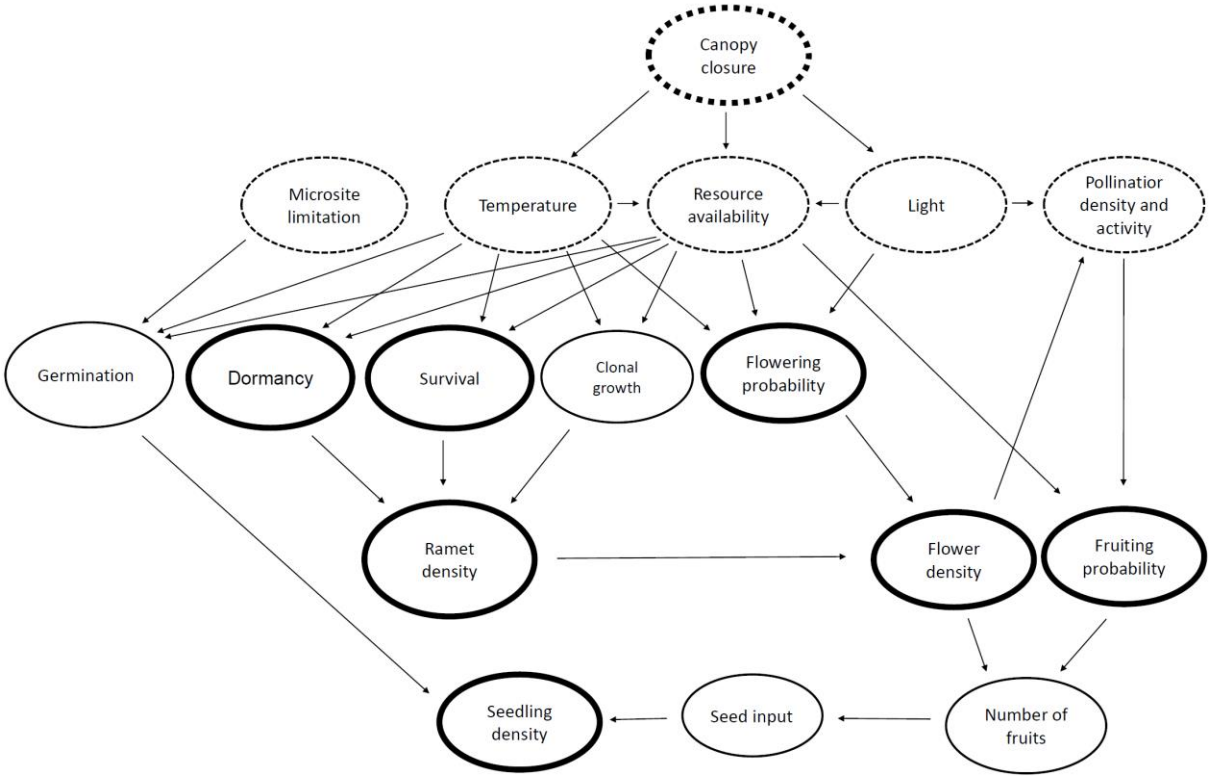

Figure 1. A flow-chart depicting the effects of canopy closure and abiotic factors (dashed line) on different demographic rates (solid lines) of forest understory herbs. Variables in bold circles are measured in the current study.

Kull, 1999; but see García *et al*., 2010) As noted in extensive field observations of this species, flowering probability, seed set, and seedling establishment are limited under closed-canopy conditions in boreal forests in northern Finland (Laitinen, 2006). Moreover, in a shading experiment, Shefferson *et al.* (2012) observed that the flower production and survival of shaded plants were lower than those of unshaded plants. These authors also noticed that orchids were able to escape the negative effects of shading through vegetative dormancy, a state in which a plant produces no above-ground shoots for one year or more, and only the below-ground rhizome survives (Shefferson *et al*., 2012).

In this study, we use long-term demographic data from northern Finland to examine whether selective tree harvest could be used as a management method for rare orchids in over-grown herb-rich forests.

METHODS

The lady's slipper orchid (*Cypripedium calceolus*) is a nectarless, clonal, long-lived herb with a horizontal rhizome. Ramets form clumps consisting of several clones, in which vegetative propagation dominates over sexual reproduction (Kull, 1995; Brzosko *et al.*, 2002). Some clones and ramets can remain dormant for several years, although one year is more typical (Brzosko, 2002; Shefferson *et al.*, 2001). One stalk supports one to two, rarely three, yellow slipper-shaped flowers. This orchid is mainly boreal, and is widely distributed from Europe to Asia. It has been declining in several countries, but many populations are now stable or increasing due to the implementation of successful conservation actions (Rankou and Bilz, 2014).

As part of the Metsähallitus' EU Life funded project in 2001 (details in Laitinen, 2006), ten lady's slipper orchid sites from northern Finland were chosen for active forest management and ten sites as controls. The habitats consisted of herb-rich forest. In the summer of 2001, a randomly located 1 × 10 m plot was established at each study site. At sites with under 100 ramets, an extra square (up to 8 m^2) was included to obtain a sufficient sample size. Each isolated ramet or ramet clump was marked using a steel stick with a numbered plastic label. We use a clump as a proxy for a

clone, as the ramets grow so closely together that we could not reliably follow them individually, and the identification of actual genetic clones would have required genetic tests or excavation of the plants.

In years 2001–2004, 2008–2010, and 2014–2016, we visited all sites once a year during the fruiting time in July and recorded the state of each ramet clump as dormant, vegetative, or flowering. The numbers of flowers and capsules were counted, and newly emerged clumps and seedlings were marked every year. Tree removal was conducted in the winter of 2001 (i.e. after the first summer survey). Three types of forest were represented in the management areas, and each differed in the intensity of selective tree harvest: (1) dense spruce forests, where half of the total tree basal area (TBA) was cut (mean of 48.9% (SD 8.4%)), (2) sparse spruce forests, where one-fourth of the spruce TBA was cut (mean reduction in TBA mean of 26.4% (SD 7.4%)), and (3) sparse broadleaf forests, where one-fourth of the total TBA was cut (mean reduction in TBA mean of 25.7% (SD 0.7%)). Control sites, in which no trees were cut, included the same forest types as the treated sites. The size of the managed areas varied from 600 m^2 to 1700 m^2, with the demography plot in the middle. Tree removal was conducted without heavy machinery and all logs and branches were removed from the sites. A thick snow cover protected the plants and ground from mechanical disturbance during cutting.

Clonal growth and sexual reproduction of the lady's slipper orchid were assessed using the following six variables: the densities of mature ramets and seedlings (per m^2), flowering probability, fruiting probability, dormancy and survival. We tested differences in demographic rates between managed and control sites using generalized linear mixed models, taking into account the starting level (the value of a given response variable in 2001 before treatment) and time period.

RESULTS AND DISCUSSION

A 25 – 50% reduction in tree basal area increased population size at the ramet-level by enhancing survival and clonal growth at sparse broadleaf forest sites, and increased the probabilities of flowering and fruiting at spruce forest sites, although these positive effects were not seen during all years (Figure.2). The positive effect of forest harvest on flowering and fruiting probabilities during the immediate post-harvest period was probably due to increased nutrient availability and increased pollinator density and activity (Figure 1) as the species is mostly pollinated by solitary bees of the genus *Andrena* that prefer open or half-open habitats (Antonelli *et al.*, 2009, Erneberg and Holm, 1999; Kull, 1999). However, the positive effects on

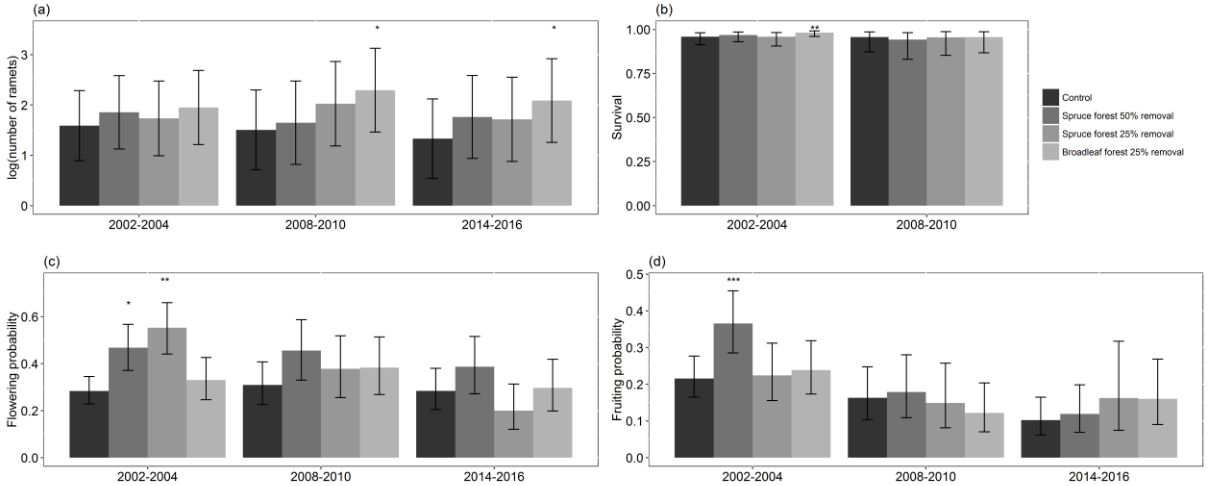

Figure 2. **(a)** Ramet density, **(b)** survival, **(c)** flowering probability, and **(d)** fruiting probability of the lady's slipper orchid by treatment and time period. Bar heights represent the model prediction, and error bars denote standard errors. Asterisks indicate a significant difference between the treatment and the control of the same time period. Predictions are averaged over geographic regions and starting level covariates. Count responses are on a logarithmic scale and proportional responses are back-transformed to the original proportion scale.

flowering and fruiting were seen only in the most intensive treatments and only during the first few years after tree removal. Furthermore, the increased capsule production did not translate into a significant increase in seedling production, suggesting that seedling production of the lady's slipper orchid is constrained by the number of favorable microsites rather than by the number of seeds (Kull, 1998). This calls into question the benefits of tree harvest for the sexual reproduction of this species.

However, tree removal appeared to increase the clonal reproduction and thus the population size at the ramet level, as the ramet density was significantly higher at the broadleaf 25% removal treatment sites than at the control sites (Figure 2.). Although this effect was seen with a lag of several years, it was persistent and still visible at the end of the study. This higher ramet density seems to be due to increased clonal growth (greater number of ramets per clone) and not, instead, the consequence of increased clump sprouting, as the treatments had no effects on the proportion of dormant clumps.

CONCLUSION

Previous observations from Scandinavia (Antonelli *et al.*, 2009; Bjørndalen, 2015) and our findings show that selective tree harvest might be a suitable management method for the lady's slipper orchid. Furthermore, while we can expect responses to change in canopy cover to be species- and habitat-specific, it is likely that tree removal could be used as a management method for other understory species whose reproduction benefits from canopy gaps.

Acknowledgements

We thank Metsähallitus for establishing and monitoring the study plots in 2001 – 2004. Especially, T. Laitinen, P. Kulmala, as well as K. Uimi conducted field work during the earlier observation years. We wish to thank also our field assistants E. Rams, A-M. Borshagovski and T. Hyttinen for valuable help during the later observation years, as well as impress our thanks to Oulanka Research Station for general assistance. This work was supported by the European Union Life funding, Metsähallitus, University of Oulu, the Jenny and Antti Wihuri Foundation (SH) and the Academy of Finland (grant 285746 to SR).

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Mosaic cycles of ecosystems and population strategies of terrestrial orchids

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Sustainable biodiversity may be achieved only by permanent natural disturbances in an ecosystem. Many species of orchids are among protected plants and need constant protection from serious human impact on natural processes. However having a stress-tolerant and ruderal population strategy, orchids are dependent on biogenic and anthropogenic factors that violate dense tree and shrub canopy as well as closed ground vegetation. Manifesting a stress-tolerant strategy, they inhabit swamps, fens and floodplains, avoiding the competition with other herbs. They are renewed by seeds on disturbed by wild boar diggings, windthrow gaps, and scree. Showing features of ruderal strategy, orchid populations expand and increase in density following surface fires. Orchids are pioneering species of abandoned quarries, dried reclamation canals, clearings, roadsides, and talus. Higher population density numbers are found in the early stages of succession. Orchids may exist for a long periods of time in low numbers, in low level of vitality and even move into a state of secondary dormancy under unfavorable conditions during the next stages of succession. The species having narrow ecological tolerance ranges may disappear during the overgrowing of meadows in the absence of grazing ungulates and haymaking. Special management practices are required in nature reserves by imitating natural processes to establish the forest and meadow mosaic patterns.

The Orchid Specialist Group at the start of the Quadrennium

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In early 2017, the Orchid Special Group (OSG) of the Species Survival Commission (IUCN) was reconstituted for the new Quadrennium (2017-2020), with *c.* 150 members around the world. This process gave us the opportunity to rethink some of the aspects of how the group should be run and what the opportunities are for how we work to conserve orchids. Red list assessments will remain a main focus for the OSG as this is a great way of raising awareness of the threats to orchids worldwide, but we can also work towards orchid conservation in other ways. Major changes leading up to the reconstitution were the establishment of two related new thematic groups, one investigating orchid trade issues and one looking at the use of novel molecular techniques for identification of orchids, particularly in processed forms such as traditional medicines and food; through these groups, we have started a dialogue with the CITES Plants Committee. We have begun to investigate the opportunities for using social media to strengthen the network of people interested in orchid conservation, e.g. the OSG Facebook group was started at the 6th International Orchid Conservation Congress (IOCC) and now has *c.* 500 members. Plans for the 7th IOCC, to be held at Kew in 2019, are underway, with the working title of "Orchid Conservation – the Next Generation" indicating a focus on the importance of involving younger conservationists in this important work and recognizing the role of next-generation sequencing technology in taking the work forwards.

Improvement of evaluation of the extinction risks of the French wild orchids using citizen science's shared data

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Almost ten years after the first French national orchid Redlist 2009' publication, a new extinction risk assessment has been carried out using citizen science data shared through the Orchisauvage Website-based project. Observers have shared in real time about 400 000 records since the launch of the project, 4 years ago, almost the same amount than the ones accumulated during the 30 years prior 2009.

In comparison with the previous assessment, the distribution obtained in this new one is slightly less dense but the quality of the information is higher thanks to a –in real time- validation process. It appears that many indicators show an increase: the distribution in altitude has extended for most species, as well as the today known range of the blooming periods, or the extent of occurrence. This may result from a better knowledge, or a consequence of the extension of the species distributions, mostly for Mediterranean species, or the decrease of others, due to threats.

Evolution of the risk extinction level using IUCN categories and criteria guidelines is therefore proposed for some species. The strengths and the consequences of using shared citizen's data are presented and discussed.

The ongoing story of Ambodiriana forest in Madagascar, a representative case study of *in situ* conservation

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Orchids cannot be protected if their habitats are not, but carefully chosen habitats allow protecting large numbers of species, this is what tells us the Ambodiriana case study, an ongoing story mixing ecological fight and scientific research.

The Ambodiriana forest, located near the village of Manompana on the East Coast of Madagascar (S 16° 40', E 49° 42', around 200 km North of Toamasina), is one of the latest coastal forests in Madagascar. Despite a rather small area, 240 hectares, it is blessed with at least hundred species of wild orchids. Among them some are considered endangered or critically endangered by IUCN, some are potentially new to science, and others are known only in this forest. Along with these orchids, rare palms, frogs and lemurs, including the aye-aye (*Daubentonia madagascariensis*) have been observed.

In 1996, slash and burn culture (tavy) approaching dangerously the site, discussions started between on one side the local community and authorities, the Malagasy government and on the other side a newly created NGO called ADEFA (Association de Défense de la Forêt d'Ambodiriana), for the conservation of this primary forest that was once protected by a "fady", i.e. a taboo. Eventually an agreement was signed in 1999, for 15 years. ADEFA was based in La Réunion island, as most of its members and its president, Chantal Misandeau, who regularly came to Ambodiriana. During 15 years, eco-tourism activity was developed, with a team of guides and bungalows in a camp, and regular scientific surveys by various specialists, botanists and zoologists, progressively revealed the exceptional value of the forest, which is a very specific habitat, due to 3 waterfalls that maintain a high humidity level. In 2015, for unclear administrative reasons, the agreement with the Malagasy government lamentably could not be renewed in time, causing ADEFA to be dissolved. Moreover an important theft occurred in the camp and in March 2016 cyclone Enawo hit the region and damaged the bungalows. Last but not least, the owners of the camp area, which was rented, wanted to recover their land and burn it to sow rice. In 2017 the situation is slowly improving, with the help of Rain Forest Trust and the local NGO Madagasikara Voakajy, who volunteered to help us set up a new agreement and status of the forest, with the goal of establishing a New Protected Area (Nouvelle Aire Protégée, NAP), and if necessary to buy the corresponding land. A new organisation was created in La Réunion, the "Association Des Amis de la Forêt d'Ambodiriana à Manompana" (ADAFAM), aiming at creating a sustainable activity of eco-tourism that will trigger a shared interest of local community to protect the forest on the long term.

The presentation will give the latest news and will present an overview of the orchid flora and other riches of this endangered but so far miraculously preserved primary forest. Orchid protection not only encompasses many topics of science, but also straddles diplomacy, law, economy and psychology!

Figure 1. *Gastrorchis tuberculosa*, one of the emblematic orchids in Ambodiriana.

Figure 2. *Gastrodia madagascariensis*, first mentioned in 1925 without flowers, then lost, recently rediscovered in Ambodiriana.

Some recent proposals in *Platanthera* (Orchidaceae) systematics in Western Europe

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Recent research on *Platanthera* in Western Europe has shed new light on the structure of sympatric populations of the two widespread species *P. bifolia* and *P. chlorantha* including intermediate looking individuals, as well as populations comprising mainly or exclusively such intermediate plants. Based on morphological, molecular and chemical arguments, it was demonstrated, in the first case, that most so-called "intermediates" are in fact representatives of *P. bifolia*, while in the second case, "non-hybrid" intermediates constitute an independent lineage, which was given the name *P. muelleri*. True hybrids between the first two species seem to be very rare. Other situations with mainly intermediate looking individuals between *P. bifolia* (s.l.) and *P. chlorantha* in Southern Italy were given the name *P. bifolia* subsp. *osca*.

Other morphological and ecological comparisons conducted in Belgium plead toward recognition of two independent taxa within *P. bifolia*. The latter name should be restricted to allopatric populations growing on acid soil, in open areas with fresh to marshy conditions. On the other hand, *P. bifolia* populations, often growing in sympatry with *P. chlorantha* as described above, on basic soil, in semi-open to shaded habitats, could be given the name *P. fornicata*.

The paper further discusses unresolved situations that would deserve additional research.

Illegal trade of wild orchids poses a serious risk to previously unthreatened species in Mexico

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In recent years illegal wildlife trade (IWT) has become more common for orchids in Mexican markets, especially in indigenous areas with high biodiversity. This activity constitutes a serious threat for populations subject to extraction, but studies on this subject only document species richness; little is known about sales volumes, species highly demand, vendor's socioeconomic profile, and factors determining incomes. These factors were studied in eight markets from Oaxaca, Mexico, where is practiced IWT for orchids. Here, most vendors were women over 25 years of age, with low schooling, speakers of an indigenous language. Fifty-six species were recorded at sell, but 97% of the total sales volume was concentrated in only eight orchids, whose prices were generally low. Price of an orchid was poorly related negatively with its abundance in the market. The demand for orchids was elastic, that is, it decreases when the price increases and vice versa. Mexican showy orchids previously not considered at risk are now threatened by IWT. We recommend a strategy that involves vendors into a sustainable management plan based on orchids highly demanded, but attending Mexican regulations as part of a policy designed to eradicate poverty in vendor's communities.

The lady's slipper orchid in France: status assessment and recommendations

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The lady's slipper is a terrestrial orchid with high patrimonial value. During the 20th century, an alarming decline was observed in Europe. In this context, we sought to establish a detailed assessment of the current situation in France. A thorough bibliographic analysis allow to review the knowledge on *Cypripedium calceolus* L. and assess the status of the French populations. In a second time, we evaluate the evolution of 36 French populations during the first decade of the 21th century. Surveys were performed in 2012 and 2013 on sites previously investigated between 1999 and 2002. Several environmental parameters were collected and analyzed statistically to identify the causes of expansion or decline. Our study results in recommendations for the conservation management of lady's slipper. To expand data collection and improve the species status assessment in France, we propose an easy-to-implement and unified long-term census protocol.

The orchid shade house network in Central-Eastern Madagascar, an effective *ex-situ* conservation tool

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Since 2011, the Missouri Botanical Garden and its collaborators have extended our Central African orchid shadehouse network to Madagascar. Four shadehouses situated in Ambatovy (two), Antananarivo and Vohibe are currently active. They have facilitated botanical inventories and *ex-situ* conservation of species found on the Ambatovy mining concession and in nearby protected areas on the eastern escarpment rainforests between 750 m and 1,100 m elevation. We have also started sampling forests at higher elevation (1,400-1,600 m) on the High Plateau. The shadehouses, currently house about 7,534 living orchid plants (around 14,500 replicates) and have yielded: 4,276 flower samples preserved in alcohol and 7,597 photographs, greatly facilitating the identification of species. Samples and living plants have been identified in more than 310 species (35 genera) of which about 80% are epiphytic orchids, 6% are facultative epiphytes and 14% terrestrial. Many orchids are threatened by deforestation and degradation of natural habitats. Rapid Red Listing based on the information available in Tropicos, the Missouri Botanical Garden's (MBG) online database, has shown that as many as 73% of Malagasy orchid species could be threatened. With new data provided by our shadehouse network, threat assessments of 160 orchid species are underway.

The adaptation of orchids to changing conditions - what in reality?

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The report discusses various possibilities for protecting orchids in the Tver region in Central Russia. An analysis is made of whether the actual conservation of species populations in specially protected natural areas is ensured. The adequacy of the identified habitats for maintaining the species within the Network of the ecological Network and the Emerald Network in Russia is assessed (for example, *Cypripedium calceolus*). Examples of conservation of orchids in temporary ecological niches created under the influence of anthropogenic activity and the stability of orchid populations to the impact of introduced species are considered. The main endangered factors in the Tver region for orchids are the disappearance of habitats (deforestation), industrial development of territories, the spread of aggressive introducent species (*Heracleum sosnowskyi*), the growth of urban agglomerations. Currently, 33 species from 20 genera of the Orchidaceae family are found on the territory of the Tver region. Over the past 100 years, 2 species have not been recorded on the territory of the region and are considered extinct (*Cypripedium guttatum, Calypso bulbosa*), 2 species have not been found for more than 70 years (*Cephalanthera longifolia, Neottianthe cucullata)*. In the Red Book of the Russian Federation (2008) 12 species (36.4%) are listed, in the Red Book of the Tver region - 24 species (72.7%). On the other hand, habitats and large populations of rare orchid species have been preserved for more than 100 years (*Cypripedium calceolus, Orchis militaris, O. ustulata*) in areas where human economic activity is conducted. In the Tver region, orchids are noted in various types of disturbed habitats: along roads and railways, in quarries, on dumps of old quarries, on deposits, in forest plantations, along meliorative ditches, in urbanized areas. A database was created using GIS.

Habitat suitability modeling for two species of Catasetinae (*Catasetum bicolor* Klotzsch. and *Catasetum ochraceum* Lindl.)

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Orchids are a species group better known by its pollination relations, however, studies related with their geographic distribution are limited. In this research, we model the potential suitable habitats for two Catasetinae species (*Catasetum bicolor* Klotzsch. and *Catasetum ochraceum* Lindl.) which have Neotropical distribution. We recognized the ecological requirements analyzing the ecological conditions in the occupied ecological niche and we identified the suitable areas for these species using Maxent V3.4.1. Occurrence records from different sources (GBIF mostly), monthly multiannual climatic data (precipitation, maximum temperature, minimum temperature, average temperature, solar radiation) and 19 bioclimatic variables from WorlClim V2 were used. With Principal Components Analysis (PCA) we selected 23 (*C. bicolor*) and 19 (*C. ochraceum*) factors among a set of 79 factors to be used in Maxent. Although the climatic requirements are different for both species, PCA shows a strong influence of temperature, solar radiation and bioclimatic factors related with the precipitation of dry periods. The habitat suitability was identified at country, biome and ecorregion scale. The results highlight the geographic space shared by these species in humid and dry tropical and subtropical forests, which are among the most threatened biomes in the world due to high deforestation rates and habitat fragmentation.

Impacts of outcross on plant recruitement in *Epipactis*

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Abstract – In many plant species, crosses between related parent leads to inbreeding depression. Few studies have been carried out in orchids on this topic. In *Platanthera*, inbreeding revealed by a reduced growth has been reported. In *Epipactis*, mating systems change from allogamy to autogamy according to the species. This genus is thus suitable to test impacts of mating systems. Selfing, crossing among plants from the same stand or from different stands and interspecific hybridization were tested. Plants of *E. atrorubens*, *E. hellerorine*, *E. muellerii*, *E. palustris* and *E. purpurata* were selfed or crossed. Fruit set was very similar whatever the cross type, including interspecific hybridization. Only a week reduced level of fruit set was observed for crosses among distant plants, which is mostly probably due to an impact of flower transportation and pollinia degradation during conservation. In *E. purpurata* and *E. muellerii*, only selfing was tested and occurred spontaneously. Seeds of *E. purpurata* were not sown. Seeds were introduced between two layers of tissues of 100µm sieve and then buried out in stands with native *Epipactis*. No seed development was observed during the first year. After the second year, some seed plots showed germination and protocorm development. Protocorm development was reported only in few replications. No seed development was reported for *E. muellerii*. In *E. atrorubens* and *E. helleborine*, protocorms with root development were noticed. Most protocorms reached few millimeters and showed occurrence of fungi pelotons. These developments were observed in all type of crosses within species. The lack of development observed in interspecific crosses can be due to the reduced number of replications. Few protocorms in both species showed a larger development with roots up to 1 cm or even more. These larger developments were only observed in seed lots obtained after crosses among plants from distant stands. These results suggest greatly an advantage in plant growth when its result from a cross of genetically different parents. The lack of difference between selfing, crossing among plants of the same stand and spontaneously produced capsules may indicated a large relationships among plants within stands and inbreeding depression. Thus orchid stand management should pay attention to the genetic structure of orchid population in order to preserve it.

INTRODUCTION

Many orchid species are threatened more or less severely by changing environmental conditions and also by genetic isolation due to population fragmentation. Importance of gene flows have been poorly investigated in the context of orchid protection. Studies in population dynamics and plant development revealed impacts of inbreeding like in *Platanthera* (Wallace, 2003). In *Epipactis*, ability to produce seeds after selfing has lready been shown by Tałałaj and Brzosko (2008). In the present study, different types of crosses, including selfing, crosses between plants of the same stand, between plants of distant stands and interspecific hybridization, have been applied on the same plants. Seed development after sowing is then observed in order to detect possible impacts of cross types in some allogamous and autogamous species of *Epipactis*.

MATERIALS AND METHODS

Plant materials: *E. atrorubens*, *E. helleborine*, *E. muelleri* and *E. palustris* plants in native stands, growing under native forest trees or poplar plantation.

Controlled crosses: protecting flower buds by a net (Figure 1), removing pollinarium after flower opening, deposing pollen on stigma for controlled pollination, protecting individual flower or inflorescence by a net (Figure 2) up to mature seed collection. Selfing (in fact geitonogamy), and controlled pollinations have been tested on different flowers of each plant and were identified by color tags. Seeds

Figure 1. Flower buds protected by a net.

Figure 2. Protection by a net of hand-pollinated flowers.

produced spontaneously were also included in the study.

Sowing: seeds were extracted from fruits or collected in the protection net, then introduced between two layers of 90 µ sieve tissue (Figure 3) and finally buried into soil close to *Epipactis* plants in late November. Each of the 20 sowing plots included replications (Figure 4).

Epipactis seed development requires several months or years (Tešitelová *et al*., 2012). Consequently, seeds were collected six months up to two years after sowing and their development was observed under stereoscopic microscope and light microscope.

Figure 3. *Epipactis* seeds on 90 µ sieve tissue during preparation for sowing.

RESULTS

Fruit set was very similar whatever the cross type; nevertheless pollen degradation could occur during transportation between distant stands inducing a slight decrease of pollination success.

No germination was observed in samples collected 6 months or one year after sowing.

Protocorm development was observed in samples collected two years after sowing for selfing and outcrosses (within and among stands) but not for interspecific hybridization. Only few seed pockets showed germination. No germination was observed for *E. muelleri* (limited number of plots).

Protocorms showed presence of mycorrhizae with hyphae. Large development was observed for *E. atrorubens* and *E.*

helleborine produced by outcross between distant stands (Figure 5).

Figure 4. Plot with several seed pockets before covering with earth.

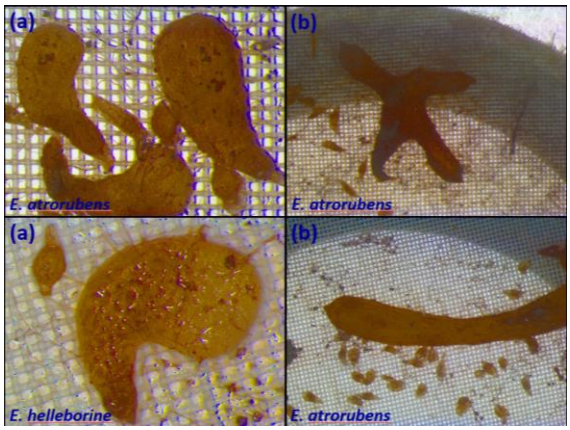

Figure 5. Protocorm development of *E. atrorubens* and *E. helleborine* two years after sowing: **(a)** seeds obtained by spontaneous pollination; **(b)** seeds obtained by crosses between stands.

CONCLUSION

Seeds buried into soil can be still found after two years and showed protocorm development in stands with native *Epipactis* plants but only few developed. Young plants have been observed in *E. atrorubens* and *E. helleborine* but not in *E. muelleri*. The influence of nematodes remains questionable.

Much more important development of protocorms have been observed in seeds derived from interpopulation crosses.

Gene flows among stands could be very important for seed recruitement and should be preserved in stand management for orchid conservation.

Acknowledgements

The present study was initiated with a support of French orchid society and two grants, one from CG38 – Isère, France and one from Botanical society of France

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Consequences of herbivory and climate for the life-history of a northern orchid, *Calypso bulbosa*

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In terrestrial orchids, individual plants do not always sprout every year, but a proportion of the population can remain belowground. This prolonged dormancy can be an adaptation to avoid adverse conditions, e.g. periodic stress or herbivore outbreaks. However, perennial plants can store and reallocate resources across years, so the effects of these environmental drivers can be difficult to detect. Functional linear models (FLM) provide a method to detect short- and long-term effects of these factors. We studied the effect of herbivory and climate factors (monthly temperature and precipitation) on the life-history of an orchid, *Calypso bulbosa*, with demographic and climate data from Finland (2002-2017). We estimated the change in the number of observed plants and vital rates (survival, emergence, flowering) by fitting FLMs with lags 0-5 years in herbivory and 0-24 months in climate factors. In the shortterm, herbivory decreased the number of observed plants and emergence. Warmer fall conditions two years prior increased survival, and one year prior increased the number of observed plants. Warmer conditions in the growing season decreased dormancy the next year. Together, these patterns suggest that dormancy increases under stressful conditions, and that plants respond to environmental drivers at varying time scales.

Can terrestrial orchids keep pace with a changing climate? A study case in Central Italy

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Many natural systems are being affected by global climate change, especially by temperature increase and drought. The effects on orchid populations are difficult to predict but evidence from our studies suggests that the recent climatic trend is already affecting the distribution and reproduction of many species in Central Italy and that some orchids are likely to be more vulnerable than others. In Northern Latium, a warming climate, together with ever more frequent and extreme droughts, is threatening with extinction some terrestrial orchids living in the so-called "*depressed beech forests*" (growing at a lower altitude than typical, 400-600 m a.s.l.). Here, the orchids with a late-spring/summer flowering result extremely vulnerable, as unable to survive the prolonged drought periods which have characterized the last years. In fact, our monitoring has highlighted a drastic loss of reproductive success in species like *Epipactis placentina*, *E. gracilis*, *E. helleborine*, *E. microphylla*, *Dactylorhiza maculata*, and *Cephalanthera rubra*, more and more often unable to reach the fruiting and seed dispersal phases, which should occur in summer. A significant biodiversity loss is foreseeable in such beech forests through local decline and extinction of orchid populations, due to the increasing temperatures and drought.

An update of the Sardinian orchids check-list

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Sardinia, together with the Greek islands, is one of the hot spots of the diversity of Mediterranean orchids. Insularity is certainly one of the factors that has most favored the formation of endemism. In this context, pathways of species colonization, genetic drift, adaptation to local conditions, and variations in the force and direction of natural selection mediated by pollinators can determine the formation of local "breeds". It is clear that the orchids, thanks to their particular anemocorous dispersion, by which light seeds are driven by the wind at great distances, may have reached the Sardinian-race platform, most likely through routes from the Tuscan archipelago, Provence, Spain and North Africa.

Once they reached the Island, orchids had to adapt to new conditions, such as the possible absence of their specific pollinators, which did not always follow the same colonization paths, the simultaneous presence of new pronouns, which offered the opportunity to occupy new ecological niches, and finally coexistence with phylogenetically similar species that have colonized the Island from other migratory routes.

Recent studies (Bateman *et al*., 2003), using ecological and molecular analyzes, have made it possible to better understand what the evolutionary dynamics of orchids have been and still are, and how they can be influenced by the anthropogenic impact. Sardinia, despite its insularity, is characterized by a particularly rich orchidological contingent confirmed by the numerous publications produced especially in the last thirty years during which new research fields have been developed that have provided interesting biosynthesics novelties.

The number of taxa currently present in Sardinia is not definable with certainty as it varies according to the taxonomic assessment that the various authors attribute to some of them. In the recent Delforge publication of 2005, Sardinia reported 71 entities compared with 63 of Scrugli and Cogoni in 1998. According to our opinion, the presence in some of these taxa on the island must be verified with greater certainty. Below are some brief critical considerations.

In the light of these data, the orchidological heritage of Sardinia amounts to 68 taxa belonging to 15 genera, of which the most numerous are *Ophrys* with 28 (42%), *Anacamptis* with 7 (11%), *Epipactis* with 6 (10%), *Orchis* with 5 (7%), *Serapias* with 4 (6%) and *Neotinea* with 4 (6%).

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Orchids of Guatemala

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Guatemala is a relatively small country in term of size, but its geographical and biological diversity is enormous. It is estimated that about 10,804 species of vascular plants can be found there, many of them are endemic. Its orchid richness is impressive and for many years poorly studied. It ranges from a little more than 100 species mentioned by Bateman (*The Orchidaceae of Mexico and Guatemala*, 1843), 527 species mentioned by Ames & Correll (*Orchids of Guatemala and Belize*, 1952-1953), 800 species proposed by Archila (1992) and 1237 proposed also by him in 2014. To understand the existence of orchid megadiversity in Guatemala it is important to consider the integration of edaphoclimatic factors as latitude, topographic relief, plate movement, volcanic chain and rain systems. About 20% of the orchid diversity in Guatemala can be found in the Department of Alta Verapaz, where c.a. 242 species, representing 60 genera, have been reported. The main aim of our study was to catalog and update the knowledge about orchids of Guatemala. The last comprehensive work about Guatemalan orchid flora has been published in 1952, where 89 genera has been reported. As our research revealed, more than 220 orchid genera occur in Guatemala.

Orchisauvage (www.orchisauvage.fr): collecting and sharing orchids records

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Orchisauvage is a participative science website developed to collect and share orchid records in order to improve knowledge and actions towards orchid conservation. User-friendly and clear ethics have allowed a huge participation with 1) more than 400 000 validated orchid records collected in less than 4 years and 2) more than 40 000 pictures shared by around 2 500 citizen "scientists". Each record gives, among other things, information on precise location supplemented by information on developmental stage. This leads to improve knowledge on French wild orchids in the fields of phenology and ecology (including concrete distribution). Analysis of the collected data is already used to update the IUCN national Redlist. The database is managed in order to avoid any threats on orchid populations. Data can be recorded in the website or through NaturaList application directly from the field. Citizen "scientists" access on real-time to various information including distribution and phenology and benefit in turn from all shared records. Further improvement are planned to offer new tools to better answer to the participants wishes and in order to develop collaboration at national and international levels.

Long term storing effect on viability and lipid acids profile of *Encyclia adenocarpa* (Lex.) Schltr. seeds

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Mexico has 6% of the Orchidaceae diversity, one of the most diverse botanic families; however, the habitat loss and the species overexploitation have diminished their populations. Seed banks could be a great option for storing *ex situ* Mexican orchids' germplasm. In this kind of storing, seeds' viability and biochemical profile should be evaluated periodically. In this project *Encyclia adenocarpa* seeds collected in 2007 and 2014, preserved in UNAM FES Iztacala seed bank were aged for 0, 6, 12 and 18 days under 45 °C and 45 % RH conditions. Seeds were germinated in Knudson C (KC) and Phytamax (PH) media (at 25 ± 1 °C with photoperiod light 12h/ dark 12h). Viability was analyzed using tetrazolium blue chloride assay, and the lipid acid profile was determined using GC-MS. Scanning electron microscopy and optic microscopy were used for examining seed morphology. Six days aged seeds grown in Knudson C media showed 95 % of germination, while wild seeds collected in 2007 did not germinate at all. Accelerated and natural ageing of seeds of *E. adenocarpa* reduced unsaturated fatty acid concentration and modified their anatomic and embryonic structure. These damages caused loss of seed viability.

A simple double-staining technique to assess seed viability in terrestrial orchids

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The need to estimate seed quality in seed-lots to optimise seed-banking efforts has stimulated researches for rapid methods to determine seed viability. Orchid seed quality is determined in various ways, especially by germination tests (even if time-consuming) and by viability tests, usually by Tetrazolium (TZ) staining. Terrestrial orchid seeds have an impermeable testa, so they require specific chemical scarification prior to vital staining to improve its effectiveness. Our results proved that the scarification protocol affects significantly both seed coat permeability and TZ results (2-way ANOVA, $P<0.0001$). In fact, eight different scarification methods resulted in TZ viability percentages ranging between 0 and 100% for the same seed-lot. Here, we report a rapid, simple-to-use protocol that can be used to test terrestrial orchid seed viability. Performing a permeability test by means of Trypan Blue dye, following the standard TZ staining, provides rapid information about seed coat permeability, colouring only the cells with corrupted membrane integrity. Viability can be calculated as the ratio between viable (bluish-to-blue testa and rose-to-red embryo) and permeable (bluish-to-blue testa) seeds so avoiding under-estimation of TZ results. Such double staining gives higher viability results than the simple TZ test, with a difference negatively correlated with the scarification effectiveness.

Seed micromorphology and morphometry of some temperate orchids (Orchidaceae)

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To reveal both qualitative and quantitative seed properties of the species belong to Turkish epidendroid and orchidoid genera in detail and to investigate which properties are diagnostic among these taxon, the seed micromorphology and morphometry of 12 taxa were examined using a light and electron microscopy. The seed micromorphological and morphometrical characteristics were examined with the help of canonical discriminant analysis and hierarchical clustering analyses by taking into consideration the taxonomic status of species belonging to the subfamilies Orchidoideae and Epidendroideae. In this study, significant differences were detected in terms of several features such as seed shape, seed length, periclinal wall ornamentation, testa cell shape, embryo length, width and volume. Two main patterns have been observed, one is similar shape and size of chalazal and medial cells, higher seed morphometrical properties and the number of cells along the longitudinal axis, for epidendroid species. The other has differences in shape and size between chalazal and medial cell, smaller seed sizes and the number of cells along the longitudinal axis, for orchidoid species. This study confirms the diagnostic value of qualitative and quantitative seed features.

This research was funded by a grant from the Scientific and Technological Research Council of Turkey (114Z702).

Mycoheterotrophy and mixotrophy in orchids: an update

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A particularity of orchids is their mycoheterotrophic germination, where reserveless seeds develop into a heterotrophic seedling, thanks to the colonization and carbon provided by a symbiotic fungus (Dearnaley *et al*., 2016). The seedling later forms green leaves in most case: the fungus, generally belonging to the polyphyletic 'rhizoctonia' aggregate (Dearnaley *et al*., 2013), then turns into a purely mycorrhizal fungus, and colonizes roots only. At this adult stage, green orchids are believed to become autotrophic and to reward the fungus with their own photosynthetic carbon, as in most other mycorrhizal associations.

However, some species rely on mycoheterotrophy at adulthood and lost photosynthesis. This evolution of non-green species occurred ca. 50 times independently in the orchid family. It was more recently realized that some green orchids, phylogenetically related to mycoheterotrophic species, although photosynthetic, are partially mycoheterotrophic, a strategy called mixotrophy (Selosse and Roy, 2009). In the later species, difference in isotopic abundance (^{13}C) between fungal and photosynthetic carbon and the examination of albinos (rare achlorophyllous variants that survive *in natura* thanks to full mycoheterotrophy) were instrumental in the elucidation of mixotrophy. Mycoheterotrophic and mixotrophic species rely on the symbiotic shifts from the usual rhizoctonia partners to totally different fungal taxa, which are either saprotrophic (in the tropics mainly) or mycorrhizal on surrounding trees (Hynson *et al.*, 2013). Moreover, mixotrophy is viewed as an evolutionary step toward mycoheterotrophy.

More recently, isotopic particularities found in most green orchids that are putatively considered autotrophic raised the possibility that they are mixotrophic as well (Selosse and Martos, 2014; Gebauer *et al*., 2016). We discuss this issue in an evolutionary perspective, and also address the limits of isotopic approaches, in order to suggest next steps in research on mixotrophy in the orchid family

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Where do orchid mycorhizal fungi come from?

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The distribution and abundance of orchid mycorrhizal fungi (OMF) can influence the the establishment and resulting spatial pattern, as well as the population dynamics, of their host plants (McCormick *et al.*, 2016; Rock-Blake *et al.*, 2017). Yet, our understanding of these mycorrhizal associations is currently limited by our restricted knowledge of the ecology and spatial distribution of OMF, especially those belonging to the 'rhizoctonia' complex *sensu lato* (McCormick and Jacquemyn, 2014).

In a recent investigation focusing on Mediterranean grassland orchids, some OMF rhizoctonias were undetected even in the soil beneath their orchid hosts, questioning the view of these fungi as unspecialized soil saprotrophs (Voyron *et al.*, 2016). Whereas members of the Sebacinales and Ceratobasidiaceae are known to establish mycorrhizal or nonmycorrhizal endophytic associations with non-orchid plants, the ecology of the Tulasnellaceae is largely understudied (Selosse, 2014; Selosse and Martos, 2014).

We will present results of an experimental manipulation aimed at assessing the impact of the surrounding non-orchid vegetation on the occurrence of OMF in the roots of the orchid host, by comparing the frequency of the fungal symbionts in the roots of naturally grown *Spiranthes spiralis* plants in either undisturbed soil cores or soil cores in which the neighbouring non-orchid plants had been manipulated or removed.

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Untangling factors underlying distribution of forest orchids

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Factors driving species distribution patterns are key topic in current ecology. This goal is rather challenging in plants, which depend on mycorrhizal fungi for germination and subsequent growth, such as orchids. In view of dispersal and habitat limitation concepts, orchids are considered little limited by seed dispersal, but highly habitat specific. In contrast to this assumption, many theoretically suitable sites stay unoccupied. Using four forest orchids differing in fungal symbionts and demands for limestone substrate, we studied drivers of their distribution in a fragmented landscape. We combined analyses of seed dispersal and population genetic structure with seed sowing in occupied and unoccupied habitats and analysis of mycorrhizal fungi in soil, adults, seedlings and ectomycorrhizal tips from surrounding trees to untangle influence of diverse environmental predictors. While 95% of seed were trapped less than six meter from mother plants, gene flow study showed effective long distance transport. Germination success of all studied species was influenced by habitat (forest type and soil pH), together with presence/absence of suitable mycorrhizal fungi on the site, and the highest germination rate occurred at occupied sites. Although seed germinated also at unoccupied sites, dispersal limitation seems to play smaller role in orchid distribution than habitat limitation.

Low within-species specificity for fungal partner in the rare mycoheterotrophic orchid *Epipogium aphyllum* Sw.

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Most mycoheterotrophic plants exhibit high specificity towards their fungal partners. Individuals associate with a narrow fungal clade or fungal species. Some within-species variation in specificity has been reported raising questions about genetic *versus* habitat influence on this trait. This question, though very relevant, is rarely explored. *Epipogium aphyllum* Sw. is temperate, fully mycoheterotrophic orchid species that associates with a great range of species within the ECM fungal genus *Inocybe*. Unlike the other studied MH orchids, it shows marked variability within and between populations in associated fungal species. Our study asks whether the observed pattern reflects low specificity or cryptic, genetically-based differences in fungal preferences. We studied the mycorrhizal associations in an abundant population of *E. aphyllum*. We sampled rhizome from 40 randomly selected individuals. Plants were genotyped using nuclear SSR and cDNA markers designed for this species, and whose polymorphism has been assessed over Europe. Fungal symbionts were barcoded using ITS marker. All the sampled individuals belonged to one multilocus genotype presumably derived from vegetative propagation. We identified 14 fungal OTU that were widely distributed into two subgenera *Inocybe* and *Mallocybe*. Obtained results point to low specificity within *Inocybe* in studied species. We discuss the evolutionary and ecological implications of this pattern.

The evolution of mycoheterotrophy in Neottieae

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Mycoheterotrophy, the nutrition based on carbon from mycorrhizal fungi, is how all orchids start their lives during the germination of their reserve-less seeds. Whereas most of them then develop leaves and become photosynthetic, some species arisen independently remain achlorophyllous and continue to feed mycoheterotrophically for their whole lifespan. Others use both photosynthetic and mycorrhizal carbon sources, a nutrition called mixotrophy. Mycoheterotrophy at adulthood has evolved several times in different orchids lineages, such as the well-studied tribe Neottieae. This group of terrestrial Epidendroideae encompasses six genera with species showing the three nutrition types described above: autotrophy, mycoheterotrophy and mixotrophy. The phylogenetical relationships between the genera has however remained unclear for long. Here we present a robust phylogenetical framework as well as newly sequenced plastidial genomes of Neottieae spp. We suggest a reconstruction of the evolutionary history of mycoheterotrophy in this group and the associated changes in chloroplast functioning. Our results reveal a continuum in plastidial genome degradation, which does not always match the level of mycoheterotrophy, suggesting asynchronous metabolic and genomic evolutions towards mycoheterotrophy in Neottieae.

The mystery of albinos orchids - a integrated 'omics' study to better understand mycoheterotrophy

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Some terrestrial photosynthetic orchids can have two carbon sources for their metabolism: photosynthesis and their mycorrhizal partners. These mixotrophic plants can adjust the relative balance of their carbon sources depending on the environmental conditions. In some rare occasions, they can even completely loose the photosynthetic ability without obvious adverse effect resulting in albino plants, which survive and eventually flower. This highlights the extraordinary versatility of their primary metabolism. To understand the mechanisms underlying this ability and the role of their mycorrhizal partners, we performed an integrated metabolomics and transcriptomic analysis of naturally-occurring albino individuals of *Cephalanthera damasomium*, *Epipactis helleborine* and *Epipactis purpurata*. Using the localizations provided by field orchidologists, mostly from the Société Française d'Orchidophilie, samples were collected in France and Luxembourg. When comparing albino plants to green counterparts from the same populations, we observed a conserved pattern for the three species. The metabolomics analysis showed a global shift of the carbon/ nitrogen balance, with aerial parts handling mainly N metabolism in albinos, as expected from previous studies on albino plants. Surprisingly, the impact on the transcriptome was very limited, supporting the idea that the physiology of these orchids is resilient and versatile, and partially independent from photosynthesis.

In situ symbiotic seed germination in *Dendrobium* spp. (Orchidaceae): implications for orchid restoration

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Abstract – Orchidaceous taxa are known for symbiotic relationships that facilitate pollination as well as mycorrhizal associations that are necessary for seed germination. To enhance seed germination and the restoration of threatened orchid species, purified fungal strains typically found in host orchid protocorms can be isolated, verified to promote seed germination and subsequently applied to seed propagation *in situ*. Here, we describe and test the application of three fungal isolates on seed germination rates for three epiphytic species of orchids: *Dendrobium aphyllum*, *D. devonianum* and *D. nobile*. Additionally, we evaluate variance in seed germination rates on the host preferences and sowing time (all three species). Seed germination was highest for *D. nobile* (13.6%) and lowest for *D. devonianum* (9.4%); *D. aphyllum* had intermediate levels of germination (10.0%). The proportion of packets that produced seedlings varied slightly among species (77.3% for *D. aphyllum*, 73.8 % for *D. devonianum* and 76.9% for *D. nobile*, respectively). Germination rates differed significantly among hosts and sowing times and thus indicates there was significant association found between seed germination of orchids and host plants on different sowing time. The percentage germination on the trunk of *B. purpurea* was significant higher than those on the trunk of *Citrus maxima* and *Camellia assamica*. Seed germination rate also differed significantly on different sowing time for each species. Overall, our results indicate that *in situ* symbiotic seed germination is possible for epiphytic orchids and recruitment may be enhanced by species-specific symbiotic fungi and ecological and demographic parameters. Our study has broad implications for restoration efforts of rare and endangered epiphytic orchid as well as their commercial production for medicinal or horticultural applications.

Keywords – *Dendrobium aphyllum*, *Dendrobium devonianum*, *Dendrobium nobile*, germination time, host specificity, restoration

INTRODUCTION

Orchids produce tiny dust-like seeds (microspermy) that require essential carbon, water and mineral nutrient supply from their obligate mycorrhizal symbionts for germination (Rasmussen and Rasmussen, 2009; Tesitelova *et al.*, 2013). Although a number of studies have investigated the mechanisms of 1) rarity and distribution (McKendrick *et al.*, 2002; Tesitelova *et al.*, 2012) from the aspect of fungal assosication; 2) symbiosis between orchid seeds and fungi (Zettler and Hofer, 1998; Stewart and Kane, 2006; Nontachaiyapoom *et al.*, 2011; Sebastian *et al.*, 2014); 3) fungal specificity (Bidartondo and Read, 2008; Sheng *et al.*, 2012; Zi *et al.*, 2014); 4) nutrition flow from fungus to the host (McKendrick *et al.*, 2000; Kuga *et al.*, 2014) and 5) gene regulation (Zhao *et al.*, 2014; Liu *et al.*, 2015; Tsai *et al.*, 2016) that contribute to seed germination, symbiotic seed germination (SSG) remains a challenge for the reintroduction and conservation of orchid species (Batty *et al.*, 2006; Bustam *et al.*, 2014).

To date, SSG for the propagation of seedlings to be used in restoration has primarily been conducted in *in vitro* conditions because both the seeds of orchid species and their associated fungi are sensitive to microhabitat variance (Zettler and Hofer, 1997;

Bruns and Read, 2000; Stewart and Kane, 2007; Park and Lee, 2013; Sathiyadash *et al.*, 2014; Tan *et al.*, 2014; Da Silva *et al.*, 2015). The fungi in SSG are usually from roots of orchid species (Zettler and Hofer, 1998; Zettler *et al.*, 2000; Stewart and Zettler, 2002; Liu *et al.*, 2010; Chutima *et al.*, 2011; Salifah *et al.*, 2011; Fracchia *et al.*, 2014; Sathiyadash *et al.*, 2014) since root-associated fungi is more accessible compared to fungal strains obtained from host protocorms which is inaccessible to pick up in nature at the initial stage of seed germination due to its tiny size and distribution haphazardness and can be time-consuming by *in situ* seed baiting technique to generate. For example, the induction of protocorms for fungal isolation can take upwards of nine months for *D. devonianum* (Zi *et al.*, 2014), seven months for *D. officinale*, *D. nobile* and *D. chrysanthum* (Wang *et al*., 2011), four months for *Cymbidium mannii* (Sheng *et al.*, 2012) and five months for *Papilionanthe teres* (Zhou and Gao, 2016). Although fungal isolation via roots is a faster technique and may facilitate an immediate need to generate orchid seedlings, a yet unresolved question in orchid biology is whether symbiotic mycorrhizal fungi (mycobionts) utilized as a carbon source by young seedlings (protocorms) are different from those utilized by the roots of adult plants (Zettler *et al.*, 2005); in other words, even fungi originated from roots can promote seed germination *in vitro*, but it isn't necessarily to stimulate seed initiation and further development in field because of narrower ecological specificity (Masuhara and Katsuya, 1994; Steinfort *et al.*, 2010). Hence, fungi supporting the symbiotic relationship in roots may not be the same fungi that promote seed germination and seedling formation: fungi isolated from protocorms and applied to seeds may result higher rates of germination (Sheng *et al.*, 2012; Zi *et al.*, 2014; Zhou and Gao, 2016; Shao *et al.*, 2017; Rasmussen and Whigham, 1993; Brundrett *et al.*, 2003; Ke *et al.*, 2007; Keel *et al.*, 2011), although previous studies are largely based on *in vitro* symbiotic germination and have rarely been evaluated *in situ* (Shao *et al.*, 2017).

Most orchid conservation efforts typically use *in vitro* asymbiotic seed germination techniques to generate seedlings for re-introduction or population augmentation of terrestrial species. Stewart and Zettler (2002) reported to establish seedlings of *Habenaria* spp. onto soil *ex vitro* by SSG. The theory of restoration-friendly cultivation using seedlings by tissue culture has been gradually applied in *D. catenatum* plantation and *D. nobile* plantation in Guangdong (Liu *et al.*, 2013, 2014), Chongqing, Guizhou, Hainan and Yunnan provinces, China (Personal observation), which benefits industrial cultivation and conservation. But there is rare report on *in situ* SSG (Shao *et al.*, 2017; Yang *et al.*, 2017). Although seedlings generated by SSG has great potential to enhance current restoration efforts in epiphytic orchid species (Wang *et al.*, 2011; Sheng *et al.*, 2012; Sathiyadash *et al.*, 2014; Zi *et al.*, 2014; Da Silva *et al.*, 2015; Khamchatra *et al.*, 2016; Zhou and Gao, 2016; Shao *et al.*, 2017; Yang *et al.*, 2017), there is a paucity of reports on restoration by *in situ* symbiotic seedlings even though its advantages for adaption to micro-environments, higher seed germination and seedling survival rates (Liu *et al.*, 2010; Shao *et al.*, 2017; Yang *et al.*, 2017).

Xishuangbanna Dai Autonomous Prefecture, Yunan Province, located in southwestern China, is a hotspot of biodiversity with > 430 orchid species (Gao *et al.*, 2014). *Dendrobium aphyllum* (Roxb.) C.E.C. Fischer, *Dendrobium devonianum* Paxt. and *Dendrobium nobile* Lindl. are commonly used in Chinese traditional herbal medicines (Bao *et al.*, 2001; Wang *et al.*, 2001). They are distributed widely in China and neighboring countries, but all the three species are on the Chinese Red List (Gao *et al.*, 2014) as endangered, rare, and extremely endangered, at least partly due to over-collection and habitat loss. Here, we describe and examine a new *in situ* SSG technique for three epiphytic *Dendrobium* species that utilizes fungi isolated from respective host protocorms. Using this technique we address the following four questions: (1) How does *in situ* seed germination rate responses for the species *Dendrobium aphyllum*, *D. devonianum* and *D. nobile*? (2) Do the seed germination rates vary among host trunks? (3) Does the sowing time influence germination rate? (4) What are the implications for *in situ* conservation and restoration of epiphytic orchid?

MATERIALS AND METHODS

Study sites

To evaluate the cultivation and restoration of endangered epiphytic orchid species, we examined on *D. aphyllum*, *D. devonianum* and *D. nobile* as our study species. All three species are epiphytic orchids on tree trunks in open forests or lithophytes on rocks in mountain valleys (c.a 500–1500 m for *D. aphyllum* and *D. nobile*, and 1100–1900 m for *D. devonianum*). All three species are widely distributed in southeastern Asian countries, such as Bhutan, India, Laos, Myanmar, Vietnam and China, including Guangxi, Guizhou, Yunnan (Chen *et al.*, 2011).

We assessed SSG of *D. aphyllum* in a mixed forest of *Citrus maxima* and *Bauhinia purpurea* in Xishuangbanna Tropical Botanical Garden (21° 54′ 53″ N, 101° 16′ 09″ E; altitude 570 m; here after referred to as XTBG). For the species *D. devonianum* and *D. nobile*, we assessed SSG at Longpa traditional tea garden of Jinghong city (Longpa) (21° 59′ 05″ N; 101° 05′ 07″ E; altitude 1150 m) which is mainly dominated by mature *Camellia assamica.* At both sites, annual precipitation due to the tropical monsoon climate ranges between 1400–1800 mm/ yr, rainy season occurs from May to October and followed by misty cool season from November to February, and March to April is the dry hot season. Annual average temperature and mean relative humidity ranges between 18–20 °C and 86–89%, respectively. All laboratory experiments were carried out at the *Ex-Situ* Conservation & Re-introduction Group, XTBG.

Seed collection, activity testing and storage

Mature, un-dehisced, open-pollinated seed capsules were collected for each of the three species. Seed capsules of *D. aphyllum* were collected from XTBG in April, 2013. Seed capsules of *D. devonianum* were collected in April, 2014 from a *Dendrobium* plantation in Tengchong County, Yunnan province and seed sets of *D. nobile* were collected from *Dendrobium* plantation in January, 2015 in Lushui County, Yunnan province. All seed capsules were sterilized in 75% (v/v) ethanol for 2 min and dried at room temperature before being opened with a sterile scalpel under aseptic conditions. The extracted seeds were transferred to airtight glass containers containing calcium chloride anhydrous. After 4 days, the seeds were stored in glass vials at - 20 °C for long-term preservation. Seeds viability was assessed using a tetrazolium test (TTC). Based on an assessment of approximately 300 seeds per species, seed viability was tested with following values: 83.4%, 79.6%, 80.4%, for *D. aphyllum*, *D. devonianum* and *D. nobile*, respectively.

Isolation of mycorrhizal fungi

Fungal strains isolated from each of our study species have been previously shown to promote SSG in all three cases. The origin, identity, storage and capacity of the fungal strain FDaI7 to promote seed germination of *D. aphyllum* has been demonstrated and tested by Zi *et al.* (2014). The fungus FDd1 isolated from protocorms near maternal plants of *D. devonianum* in July 2012 has been shown to enhance *in vitro* seed germination for the same species (Huang *et al*., 2018). And the isolate JC-01 obtained from protocorms of *D. nobile* through *in situ* baiting techniques promotes *in vitro* seed germination of *D. nobile* (unpublished data). These strains were used to test *in situ* SSG at sites mentioned above.

Preparation of inoculated seed packets

For each treatment, we modified the methods of plastic wrap + packet in Shao *et al*. study (2017) that was shown to significantly promote seed germination of *D. devonianum* in field. Specifically, we used plastic wrap to secure a paper package containing a mix of fungal powder and orchid seeds to host trees. Prior to packet preparation, each fungal strain was cultured separately in 10–50 conical flasks (500 mL) with 100 mL sterilized potato dextrose broth (PDB) using a shaker (ZQZY-A, Shanghai Zhichu Instruments Co., Ltd., China) at 150 rpm and 25° C for 7 days. Fermented mycelia were filtered by medical gauze and washed 3 times with sterile distilled water to avoid dextrose residues (Shao *et al.*, 2017). One gram of fresh mycelium mixed with approximately 5 g agar was homogenized by blender and then dried at 30° C for two hours to maintain moderate humidity (Figure 1A). Because low seed density has been shown to facilitate relatively high percentage of germination for the species *D. devonianum* (Shao *et al.*, 2017), a certain amount of the seeds using a ear pick were added and stirred

to obtain a homogeneous mixture. Control test suggested that protocorms or seedlings could be formed only with the presence of symbiotic fungi in Shao *et al.* study (2017), so no further control tests had been replicated here. The mixture ca. 0.02 g was placed into each paper packet using spatula (Figure 1A) and then approximately one gram of sphagnum moss was added. Seed quantity in a packet was recorded by dissolving the mixture without sphagnum in 10 mL 0.1% agar solution and counted seed number under stereo microscope by placing 200 µL on glass slides using a pipette. This procedure was replicated 3 times per suspension and the seed quantity was calculated (Table 1).

Sowing treatment

Once the seed packets prepared for each species, they were watered and fixed to the trunks of host species using a plastic wrap (Figure 1B). The seed packets of different sowing time could not be placed on the same trunk because of seedling colonization at the same site from previous plantation. To assess the effect of host preference on percentage of germination in *D. aphyllum*, two hosts *B. purpurea* (approximately 8 individuals/ tree; N=300) and *C. maxima* (approximately 16 individuals/ tree; N = 718) were chosen. Seed germination was conducted at two sites for *D. devonianum* (such as Longpa and Yiwu) to test the variation on seed germination between two sites (Table 1). Four sowing trials for *D. aphyllum*, *D. devonianum* and three sowing trials for *D. nobile* were conducted at different dates (Total N = 1376) and used to estimate the influence of sowing time on percentage seed germinatio presented in the Table 1. Plantation of *D. devonianum* and *D. nobile* were conducted on the trunks of *C. assamica* at Longpa site for four and three times, which included: Arpil 22, 2015 (N = 42), May 21, 2015 (N = 109), August 6, 2015 (N = 35) and March 26, 2016 (N = 38) for *D. devonianum*; October 12, 2015 (N = 56), March 25, 2016 (N = 43) and April 28, 2016 (N = 35) for *D. nobile* (Table 1).

Statistical analysis of seed germination rate

At the date of each sowing array after 3 months, the number of seedlings (few protocorms) for each treatment was counted and the percentage of germination was calculated as (the number of seedlings/ the number of viable seeds sown) x 100. The effect of species, host, site and sowing time on the percentage of germination rate (%) were analysed by Kruskal-Wallis chi-square test. All the statitisical analyses were performed by SPSS software version 22 (SPSS Inc., Chicago, USA).

RESULTS

Germination rates among species and hosts

Overall, seed germination rate of *D. aphyllum* for the 1018 replicates across both hosts varied from 0–59.9% (10.0 ± 9.63%). Percentage of germination of *D. devonianum*

Figure 1. Fungal powder mixed with seeds for sowing: **A**, fungal powder of FDdI7 for packaging by a spatula; **B**, tea packets fixed by plastic wrap on the trunk of *Camellia assamica.*

Table 1. Effect of sowing time (dates) on seed germination in of *D. aphyllum*, *D. devonianum* and *D. nobile* (the values provided with factor effect are Kruskal-Wallis chi-squared statistic and the associated significance values); significant level: *** $p<0.0001$; * $p < 0.01$.

Species	Sowing time	N	Seed number	df	Kruskal-Wallis chi square test
D. aphyllum	23 Oct 2015	2408	44	3	44.986 ***
	29 Oct 2015	210	53		
	6 Nov 2015	424	38		
	20 Nov 2015	144	36		
Total N		1018			
D. devonianum	22 Apr 2015	42	232	3	41.783 ***
	21 May 2015	109	87		
	6 Aug 2015	35	71		
	26 Mar 2016	38	40		
Total N		224			
D. nobile	10 Dec 2015	56	43	2	8.143 *
	25 Mar 2016	43	59		
	28 Apr 2016	35	11		
Total N		134			

for the 224 replicates and of *D. nobile* for the 134 replicates ranged between 0–36.1% (9.4 ± 9.8%) and 0–63.6% (13.6 ± 14.9%), respectively. The percentage of packets that produced seedlings of *D. aphyllum*, *D. devonianum* and *D. nobile* was 77.3%, 73.8%, 76.9%, respectively.

There was no significant differences found on seed germination among three *Dendrobium* species (Kruskal-Wallis chi-squared = 4.78; df = 2; $p = 0.092$; Figure 2).

Effect of host and sowing time

In spite of the lack of species effect on seed germination, there were significant differences found with host plants (Kruskal-Wallis chi-squared = 50.66; df = 2, $p < 0.0001$; Figure 3). Besides, the sowing time showed significant differences on the percentage of seed germination of *Dendrobium* species (Kruskal-Wallis chi-squared = 104.79; df = 10, $p < 0.0001$; Table 1).

In *D. aphyllum*, the percentage of seed germination showed significant association and differences according to the host plant, *Bauhinia purpurea* and *Citrus maxima* (Kruskal-Wallis chi-squared = 52.05; df = 3; $p < 0.0001$).

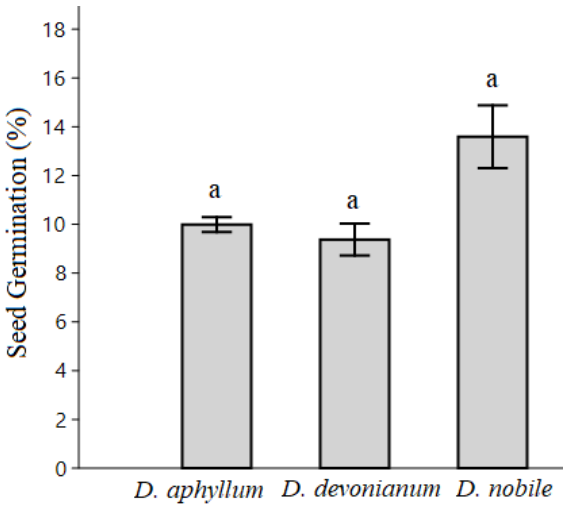

Figure 2. *In situ* symbiotic seed germination rate of *D. aphyllum*, *D. devonianum* and *D. nobile*; the same letters above bars indicate no significant differences among species according to the Kruskal-Wallis rank sum test**.**

In addition, the sowing time (dates) showed significant differences on seed germination of *D. aphyllum* (Kruskal-Wallis chi-squared = 44.99; df = 3; P < 0.0001; Table 1). Similarly, this effect is significant on both

host plants, *Bauhinia purpurea* (Kruskal-Wallis chi-squared = 14.5; df = 2; p = 0.001) and *Citrus maxima* (Kruskal-Wallis chi-squared = 28.5; df = 3; p = 0.0001). The percentage of seed germination showed significant differences of *D. devonianum* and *D. nobile* on different sowing times (Kruskal-Wallis chi-squared = 41.78; df =3; $p < 0.0001$; Kruskal-Wallis chi-squared = 8.14; df = 2; p = 0.017; Table 1) respectively.

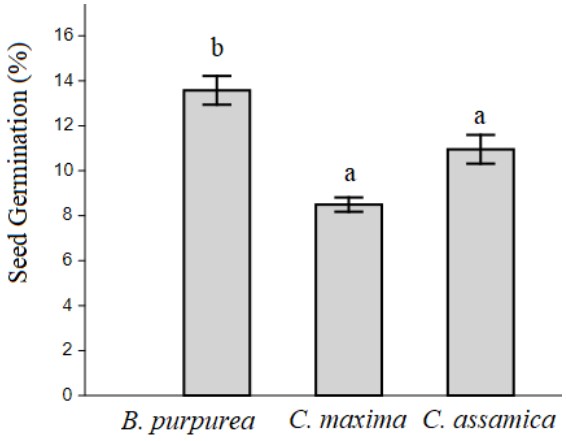

Figure 3. The effect of host plants on *in situ* symbiotic seed germination (%) for three *Dendrobium* species; small letter 'b' indicates significant different on seed germination according to the Kruskal-Wallis rank sum test.

DISCUSSION

In situ seed germination rates

The treatment plastic wrap + packet (paper) mixed with seeds, fungal powder and sphagnum proved an effective method for seed germination *in situ*. In the current study, we adjusted our methods from those of previous studies (Shao *et al*., 2017) to account for the potential loss and drying out seeds sown *in situ*. As a result, the germination rate of *D. devonianum* by this new treatment increased from ~1.4 ± 0.4% in Longpa and 0.9 ± 0.4% in Yiwu (Shao *et al*., 2017) to 9.4 ± 9.8% in this experiment.

The efficiency of the new technique varied among species. In the current study, *D. devonianum* showed the lowest mean percentage germination (9.3%), which was still far much higher than rates achieved for *D. devonianum* in previous work (Shao *et al.*, 2017). Although the germination percentage in this study is obviously lower than that in Zi and Gao's trails in which seeds grown to protocorms with percentage 44.4% and further developed to seedlings with rate 42.9% when co-cultured with the same fungus FDd1 (Zi and Gao, 2014), their trial was conducted on sterile artificial substrate under controllable conditions but not in the field. Yang *et al.* (2017) test impacts of host trees and sowing conditions on seed germination of *Dendrobium sinense* and indicated germination rate was lower than 1.0% in most cases and rarely up to 9.6% under *ex situ* conditions on preferential host tree substrate. Percentage germination was not kept stable in Yang *et al.* study since no symbiotic fungi were inoculated. The unreliable germination process may be caused by opportunistic concurrence between seeds and specific fungi, which contributed the conclusion that *in situ* a positive correlation was found between the seed germination rate and the distance of the seeds from the adult *D. sinense*. Aewsakul *et al.* (2013) achieved higher germination rates, up to 67.6%, in *Spathoglottis plicata* under sterile commercial cultivation substrates, consisting of potting soil, coir dust, and peat moss, which push the method forwards for practical application compared to *in vitro* SSG on OMA (oat meal agar) for its economical facilities and uninoculated conditions for mass seedling propagation and horticultural purposes (Quay et al., 1995). *Ex vitro* SSG of *Papilionanthe teres* by Zhou and Gao (2016) with different bark substrates without sterilization and inoculation showed 0.42% seeds developed to protocorms (stage 3) on preferential host barks and no further development at 45 days after sowing; however, 47.5% seeds cocultured with compatible fungus *in vitro* formed seedlings (stage 4) on the same host substrate at the times. Even though the germination rate decreased from *in vitro* SSG via *ex vitro* SSG to *in situ* seed sowing; seedling process in this study showed practice and potential to population construction of other epiphytic *Dendrobium* spp. in the field.

Host Preference

Although significance exists between two hosts, both showed higher percentage reports in field up to 13.6% and 8.5%, respectively, which imply certain colonial preference on the host of *B. purpurea* possibly due to its roughness, capability of maintaining moisture

and other potential chemical and physician characteristics, such as pH, nitrogen and carbon components. Harshani *et al*. (2013) suggest the prohibition of seed germination of *D. aphyllum* may be interacted by the chemical composition of the non-host barks. Some other factors such as the water holding capacity of the bark, bark stability, presence of facilitators and microclimate also may contribute to the distribution of *D. aphyllum* (Rafter *et al*., 2016). Zhou and Gao (2016) disclosed preference of *Papilionanthe teres* seed germination on three host plants (*Averrhoa carambola*; *Lagerstroemia villosa*, *Callistemon rigidus*) and no germination on bark substrates of *Butea monosperma*. The inclination was also shown with significant differences in the average fresh weight of a single germinated seed, average developmental stages, and percent of seed number with developmental stage 4. Host preference was also exhibited in Yang *et al*. (2017) studies in which *in situ* seed germination successfully occurred with the highest germination rates on three host tree species with *Dendrobium sinense* but failed to germinate *in situ* on *Cyclobalanopsis blakei* and regardless of setting (*ex situ* or *in situ*) on *Exbucklandia tonkinensi*, the latter two hosts with no associations to *D. sinense*.

Sowing time

Time is an important factor influencing germination in three species by temperature and air humidity. Germination rates varied significantly among sowing times for each of the three species screened in this study. All the sowing times of *D. aphyllum* belonged to the misty cool season and in proximity, however, similar significant difference was found among trials on the trunk of *B. purpurea* and of *C. maxima*, which was possibly caused by microhabitat and other factors. Significant differences on seed germination trials of *D. devonianum* and *D. nobile* were found at different times which might be probably caused by climate factors in three seasons, including moisture and temperature in microhabitat.

Orchid seed germination is in some cases strictly seasonal, employing a short time window between seed ripening and seed vitality loss, as the seedling stages before the development of leaves and storage tissues are highly dependent on sustained moisture and appropriate thermal regimes, synchrony of germination and time and conditions for sufficient growth before an unfavorable season can be critical for germination and establishment (Rasmussen *et al*. 2015). Some cases showed the key season to seed germination: Wang *et al*. (2011) harvested packets of *in situ* seed baiting in June 2008 in order to con-occur with the growing season; orchid seeds in Madagascar dispersed during the dry season would not have such access to water and would probably need to undergo a dormant period and wait for the subsequent wet season for germination (Rafter *et al*., 2016); Seeds of *Dendrobium sinense* sown *ex situ* and *in situ* had the highest rates of germination in July, coincided with the raining season in Hainan during which the fruits of *D. sinense* dehisce from May to July and high rainfall and humidity prevails (Yang *et al*. 2017); the season of seed sowing of *D. devonianum* was considered as an important factor influencing seed germination rate, which is proved by the significantly highest seed germination during the misty cool season (*Shao et al*., 2017).

Conservation implications

SSG can be used to study fungal activity and investigate the effect of fungal ecological distribution on host dynamics for terrestrial orchids by burying the seed packets. There are seldom references to apply the packets with seed and fungi to promote seed germination for terrestrials or epiphytes in the field. Before this study, at least two obstacles limit the development of SSG. One is the fact that, when the seeds germinate and form seedlings a period after introducing, it will be constrained in the nylon net packets and cannot grow out through it. The other problem is the difficulty in dealing with the fungi. It is an issue to keep seeds and fungi together during germination stages and the appropriate proportion for substantial balance between seeds and fungi. For epiphytic orchids, how to retain the moisture is also problematic but crucial to seed germination. By the new methods, all the obstacles above can be resolved. All the three species showed the potential reflected by relatively high germination rate although variable repeats.

The technique is novel and applicable for *Dendrobium* species' conservation through *in situ* cultivation and restoration. Nylon net packets are replaced by degradable tea packets

so that the seedlings can grow out the bags naturally. Sphagnum contained in the packets with seeds and fungi are rolled and covered by plastic wrap to keep moisture during the early germination stage even in the dry season. Fungal mycelium mixed with agar was made into powder by blender rather than suspension, therefore the number of seeds and powder is controllable in each packet which can be easy preserved in fridge and operated during the procedure of sowing.

However, a prerequisite must be emphasized that fungal origin and effectiveness should be originated from corresponding protocorms and proved. Although many fungal strains observed by trials promoted orchid seed germination, it is also important to question the compatibility between the fungi and orchid seedlings development (Nontachaiyapoom *et al*., 2011) in nature. Based on the previous work (Rasmussen and Whigham, 1993; Wang *et al*., 2011; Zi *et al*., 2014), we predict the best method to obtain the fungal strains from corresponding orchid protocorms formed in nature or by *in situ*/ *ex situ* baiting but not plants roots-associated and heterogeneous protocorms because specificity is higher in the field than that *in vitro* symbiotic germination (McKendrick *et al*., 2002). The right origin could initiate seed germination and support seedling further development. An example in case is fungi from protocorms of *Cymbidium manii* which can support seed germination of *Dendrobium aphyllum* but not plantlets augmentation (Zi *et al*., 2014). The role of fungal specificity remains elusive and needs to be tested. In addition, given the fact that tiny seeds and microorganism are susceptible to the microhabitat, several factors effect on the seed germination percentage, including sowing time and/ or host, should be paid much more attention.

Future directions

We propose that the populations can be constructed after two or three years' growth using SSG by introducing seeds with effective fungus/fungi directly on the trunks, the fresh stems used as Chinese medicinal herbs have the same quality to the wild plants, which can be applied for the industrial plantation. The seeds germinated and formed protocorms 30 days (Figure 4D) after sowing and further developed to plantlets at 60 days (Figure 4A, B). Puncturing a hole in the plastic wrap to help seedlings grow out of the plastic wrap is the last step and then the seedlings grow naturally without management (Figure 4C). The plantlets survived by forming pseudostems during the winter and sprouted at the beginning of the next spring. Profit-motivated conservation can benefit local people through such a friendly cultivation method and reducing dependency on the over/ illegal collection of wild plant resources. The method will contribute to *in situ* SSG for rare and endangered epiphytic orchid reintroduction and conservation.

Acknowledgments

The authors would like to thank Dr. Hélène Vogt-Schilb for valuable suggestions in manuscript. This work was supported by the National Natural Science Foundation of China (No. 2017YFC0505204) and the CAS 135 program (No. 2017XTBG-F05). We are also grateful to Dr. Qiang Liu, Ms. Dan Wang and Ya-Ting Li for laboratory preparation and field sowing.

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Figure 4. Seedlings of *Dendrobium* spp. from *in situ* symbiotic seed germination by seed packets A) and B) Seedlings of *D. aphyllum* were constrained without punctured holes even 90 days after sowing on the trunk of *Bauhinia purpurea* and *Citrus maxima*, respectively; C) Seedlings of *D. devonianu*m on the trunk of *Camellia assamica* in Longpa four months after sowing; D) Seedlings of *D. nobile* grew out of punctured holes on the trunk of *Camellia assamica* in Longpa after 45 days.

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Pollination efficiency and the evolution of specialized deceptive pollination systems in orchids

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Deceptive pollination strategies evolved independently in many orchid clades and in different geographic areas across all the continents. In these systems, plant-pollinator interactions range from highly specialised (sexually deceptive species) to generalised (food-deceptive species) and their employment can have dramatic consequences on patterns of reproductive success and on population-level genetic variation. In a survey of terrestrial orchids from the Mediterranean and Australia highly specialised orchids were consistently found to have a more efficient pollen transfer. Contrarily to highly specialised systems, in generalised pollination strategies a positive correlation was observed between flower number and pollination success. Thus highly specialised pollination systems, such as sexual deception, may allow the production of inflorescences with fewer flowers that permits the plant to allocate fewer resources to floral displays. This trade-off can be particularly relevant in water-deprived habitats and might explain the higher frequency of sexually deceptive species in these environments compared to tropical regions.

Pollination strategies in Neotropical genus *Maxillaria sensu lato* – Chemical and micromorphological analysis of floral attractants and their potential biological implications

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About 56% of *Maxillaria* representatives attract their pollinators by "empty promises" - combinations of visual, tactile and olfactory stimuli. The remainders offer different types of rewards, e.g. nectar and wax-like substances. Waxes and lipid secretions are produced usually by floral papillae and trichomes on the lip surface.

Our main aim was to investigate floral attractants in three taxa from distinctive alliances of broadly defined genus *Maxillaria*. All species were examined by means of scanning and transmission electron microscopy in search of micromorphological structures and evidences of secretion. Surface waxes and fragrance compounds were analyzed in dichloromethane extracts from whole flowers using gas chromatography-mass spectrometry (GC-MS), while nectar composition was studied in methanolic extracts from whole flowers by nuclear magnetic resonance spectroscopy (NMR). Volatiles detected included several standard monoterpenes, with the most abundant limonene in all plant species. Surface waxes were composed of saturated and unsaturated hydrocarbons and fatty acids (including atypical long-chain monounsaturated fatty acids in range of 18-28 carbon atoms), monoacylglycerols and smaller amounts of other organic compounds. Their composition depended on the plant species studied and their possible functions remains unknown, while it is supposed that they can act as protective substances against microbial infestation.

Ecological factors affecting of fruit set among Euro-Mediterranean orchids

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Fruit set of plants is an important demographic trait, since it reflects the efficiency of the pollination strategies and it is frequently used as a proxy of plant fitness in evolutionary ecology. Despite the increasing interest of reproductive success, the effect of ecological strategies on plant reproduction remains poorly documented hitherto especially at the scale of a family. Euro-Mediterranean orchids exhibit complex combinations of reproductive and ecological strategies, even at the scale of their different genera. We built here an original database on values of fruit set of 170 Euro-Mediterranean orchid species and we identified different ecological and environmental factors affecting this trait along a large spatial scale. This database is composed of more than 2250 observations of fruit set in natural populations, coming both from published and grey literature, and from observations made by orchid specialists. Nectar production, number and size of flowers, and opening up of the environment were revealed here as the most influent factors on fruit set. Despite potential influences by seed set and efficiency of germination, such information on fruit set allows comparative analysis on functional ecology and provide relevant information for population dynamics and conservation.

Do we really know orchid symbionts' behavior?

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The basidiomycete *T. calospora* (Boud.) Juel is generally regarded as an unspecialized soil saprotroph when it is not in association with orchid hosts. However, a recent survey on the fine-scale spatial distribution of orchid mycorrhizal fungi in two orchid-rich Mediterranean grassland soils investigates this aspect (Voyron *et al*., 2017). In this study we took a qPCR approach targeting the expression level of different carbohydrate-active enzymes (CAZymes), to address the actual saprotrophic abilities of this fungus under different conditions such as free living mycelium on oat-agar medium, free living on liquid modified Melin-Norkrans deprived of any source of C but enriched with amino acids, free living on sterilized litter, symbiont in orchid (*Cattleya purpurata* (Lindl. & Paxton) Rollisson ex Lindl.) roots, and an apparent saprotrophic condition in dead protocorms. The most interesting genes, among the more representative CAZyme families, were selected from a previously obtained transcriptome (Kohler *et al*., 2015). After RNA extractions and processing, RT-qPCR has been performed and results suggest that CAZymes expression may actually follow orchid living-cycle and that *T. calospora* may actually just have an occasional weak saprotrophic activity during orchid estivation.

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Does the mycorrhizal fungi distribution limit the orchid establishment in restored meadows?

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In the Czech Republic, vast areas of former species-rich grasslands converted into arable land are undergoing restoration using seeding of regional plant species and regular mowing. Recovery of species with complicated life cycles, such as orchids, is particularly slow though the seed source populations grow in close vicinity. Orchids are obligately mycorrhizal plants which need mycorrhizal fungi (mainly saprotrophic fungi from Tulasnellaceae, Ceratobasidiaceae or Serendipitaceae) for germination. We focused on seven both rare and common orchid species and investigated (i) *in situ* germination and (ii) in vitro germination and specificity. Only two rather common orchid species (*Neottia ovata* and *Gymnadenia conopsea*) germinated in the restored grasslands while others germinated only in undisturbed natural grasslands with established orchid populations. *Gymnadenia conopsea* showed also lowest specificity to mycorrhizal fungi during in vitro germination. Thus, diversity of orchid mycorrhizal fungi in restored habitats may be one of the factors limiting the establishment of some orchid species. The slow restoration of orchid species due to absence of appropriate fungi could be potentially overwhelmed by targeted introduction of fungi and substrate amelioration of restored sites.

Symbiotic seed germination and seedling growth promoted by *Rhizoctonia* fungi in *Cymbidium mastersii*, an endangered orchid species endemic to Southwest of China

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Abstract – The presence of appropriate fungal mycobiont for seedling recruitment and plant nutritional support is essential for the long-term survival of orchids in managed or restored habitats. In order to screen mycorrhiza fungus that can promote the seed germination and form the symbiosis with *Cymbidium mastersii* Griff. ex Lindl and scale produce high quality seedling, we first symbiosis cultivated seeds with different fungi in the medium of cortices symbiotic culture, then measured the biomass of seedlings, re-separation of fungus strain, studied the 3D hypha net by using the optical microscope and electron microscope, and determined the seed vitality with TTC method. The results showed that strain CLB111 and MLX102 that were separated from roots of *Cymbidium goeringii* Rchb f. and *C. sinense* Willd can promote the seed germination of *Cymbidium mastersii*. The differences of germination rates between treatments with strain symbiosises and control were significant. Re-separations from the culture medium confirmed the strains in symbiosis roots were the same as the inoculated strains. The optical microscope and electron microscope observation found that many fungus hypha infected into embryo cell and formed the 3D hypha net, and the embryo started differentiation.TTC measurement showed that the seed had high vitality. No strain was found by re-separation from culture medium, no hypha was observed and very low vitality under control treatment. Thus, it can be concluded that strain CLB111 and MLX102 can form the symbiosis and promote the seed germination of *Cymbidium mastersii*. The experiments found the fungus separated from adult orchids and the fungus that promote the seed germination were the same strains in *Cymbidium mastersii*. This phenomenon was different from the *Gastrodia elata* Blume. It was also confirmed that funguses that can form symbiosis with and promote the seed germination of *Cymbidium mastersii* were not absolutely specific. Under certain condition, different strains can significantly promote germination of one orchid species. It may need further investigation to verify the differences under different ecological conditions.

Key words – *Cymbidium mastersii* Griff. Ex Lindl, Mycorrhiza fungus, symbiosis system, 3D hypha net, seed activity.

INTRODUCTION

Cymbidium mastersii Griff. ex Lindl. produced in Yunnan and the Himalayan region. The flowers with almond fragrance bloom from September to December in each year. It is a traditional cultivated species in China with unique charm. Because of its high historical culture and ornamental value, it is still a popular collection and ornamental variety. However at present, plant division are still the only way of reproduction which is long growing period, low reproduction coefficient. The wild resources of the *C. mastersii* are on the verge of extinction because of the manual excavation and destruction. Therefore, it is urgent to study the effective germination and growth mechanism of *C. mastersii*. The orchids is a unique mycorrhizal plant. At present, almost all orchid plants need to form

mycorrhizal and symbiotic relationship with the partner corresponding fungi during their growth and development (Huijin *et al*., 2007). The seeds of Orchidaceae are tiny, the average thousand seeds are only 1-10 mg, and the seeds have no endosperm and only have the original undeveloped. The nutrient stored by the cells for seed germination is very little, so it is very difficult to germinate (Lu *et al*., 2005) under natural conditions. The results showed that only when infected by suitable fungi and symbiotic, could the seeds germinate (Wu *et al*., 2010; Arditti, 1995) under wild conditions. With the periodic dissolution of mycelium (Wu *et al*., 2005), the new mycelium infects and colonizes again, which maintains a good symbiotic relationship and provides essential nutrients for the seed germination of orchid without endosperm.

MATERIALS AND METHODS

Plant material

Seedlings of *C. mastersii* were collected from their native locality near Himalayan region of Baoshan district of Yunnan, China. Capsules were obtained through hand pollination in mother plants maintained in the field of Baoshan and collected after 12 months of pollination. At this time, the capsules were fully mature carrying green brown seeds.

Isolation of the fungus

Mycorrhizal fungi were isolated from the collected roots according to the method described in Warcup and Talbot (1967), with slight modifications. Briefly, the orchid root surfaces were first washed with tap water to get rid of soil particles and organic debris. The roots were then sterilized by immersing them in 70% ethanol for 1 min, followed by submerging them in a sodium hypochlorite solution containing 1% chlorine for 1 min, and finally by immersing them in mercuric chloride (0.1%) for 5-6 min. A piece of the sterilized root from each plant, approximately 5-6 mm in length, was put into 1 mL of sterilized distilled water in a Petri dish (9 cm in diameter) and crushed with a sterilized glass rod to disperse intracellular fungal hyphal coils (pelotons). About 20 mL of sterilized potato dextrose agar (PDA, pH 5.6) medium was cooled to 40-45 C, poured into the Petri dish, and mixed with the crushed roots through gentle shaking. The mixture was incubated at 25° C in the dark for 3-4 d. To prevent bacterial growth, the antibiotic chloramphenicol (final concentration 50 mg/mL) was added to the culture medium. Fungal colonies of consistent appearance that grew from a peloton were transferred onto fresh PDA medium using a sterilized scalpel and cultured individually. To further purify the cultures, small pieces of agar containing hyphal tips from each isolate were subcultured two more times. These cultures were then observed under a light microscope (400×) (Figure 1a, b) and putatively identified on criteria established for other orchid mycorrhizal fungi using hyphal morphology in culture (Sneh *et al*., 1991; Currah *et al*., 1997).

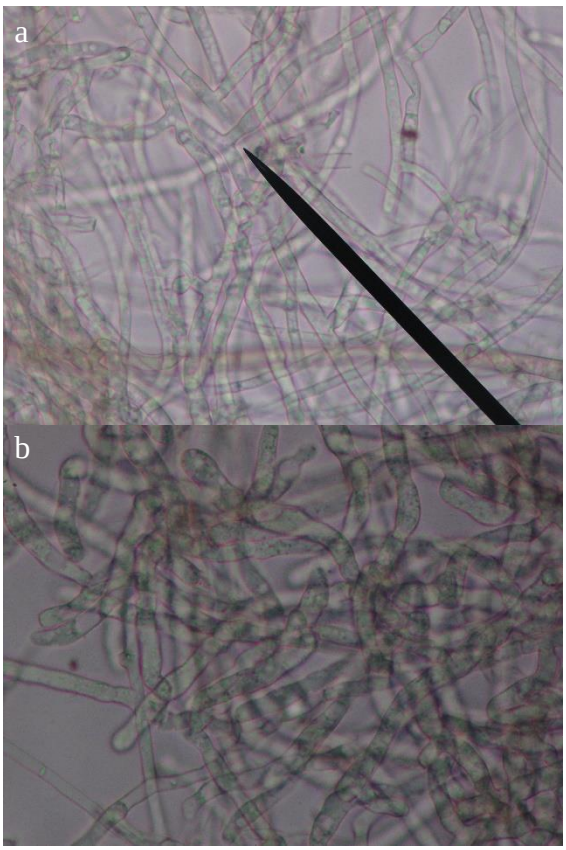

Figure1. a, **b**: The morphological feature of MLX102 and CLB111.

Characterization and identification of fungus

The fungal isolates were first characterized morphologically using the methods outlined by Currah *et al*. (1997). The fungal isolates were cultured on 1/5th PDA at 25 ± 3° C. For fast-growing fungal isolates, diameters of three colonies were measured

every 2 days until it reached 9 cm. For slow growing fungal isolates, diameters of three colonies were measured every 3 days for at least 2 weeks. Cultural characteristics (e.g., colony color, colony zonation, and types of hyphae) were observed over one month period and monilioid cells developed during the period were observed under a phase contrast microscope and photographs were taken.

Their molecular identifications were based on their DNA sequences at the internal transcribed spacer (ITS) regions of the nuclear ribosomal rRNA gene cluster. Briefly, the strains were first cultured on YEPD agar medium (per litre of medium: 10 g yeast extract; 20 g peptone; 20 g dextrose; 20 g agar; in 1 L of water) for 7 d. Mycelia from the top of the agar were harvested and ground into a fine powder in liquid nitrogen using a micropipette tip in a 1.5 mL microcentrifuge tube. Subsequent steps of the DNA extraction followed those described by Xu *et al*. (2000). The ITS regions were amplified from the extracted DNA by the polymerase chain reaction (PCR) with primers ITS1F (5'-CTTGGTCATTTAGAGGAAGTAA-3', Gardes and Bruns, 1993) and ITS4 (5'-TCCTCCGCTTATTGATATGC-3', White *et al*., 1990). A typical PCR reaction contained 5 µL of template DNA solution (*20 ng), 0.75 U of the Taq DNA polymerase, 0.25 mmol/L of each primer, 200 mmol/L of each deoxyribonucleotide triphosphate, and 3 µL of PCR buffer in a total volume of 30 µL. The reaction was performed using the following conditions: an initial denaturation step at 94 °C for 5 min, a subsequent step of 35 cycles at 94 °C for 30 s, 50 °C for 30 s, and 72 °C for 2 min, and a final elongation step at 72° C for 5 min. PCR products were purified using the MicroCLEAN kit 2MCL-10 following the manufacturer's instructions (DiaMed, Mississauga, Ontario, Canada). The purified DNA fragments were sequenced with an ABI 3700 DNA analyzer and an ABI BigDye3.1 terminator cycle sequencing kit (Huada gene, Shenzhen). The same ITS1F and ITS4 primers described above for PCR were used for the sequencing reactions. The sequences from the four strains were compared with those in the GenBank through BLAST searches. Our BLAST searches identified that their close matches were all from the broad *Rhizoctonia* fungi.

Symbiotic seed germination

The effects of fungal isolates on promoting *C. mastersii* symbiotic seed germination *in vitro* were evaluated using a modified method of Stewart and Kane (2006). Indehiscent mature capsules of *C. mastersii* (12 months after pollination) produced through hand pollination in mother plants maintained in the field of Baoshan were collected, brought to the laboratory and processed without any storage. The capsules were washed thoroughly in running tap water using a commercial detergent and surface sterilized thrice by dipping in spirit followed by flaming. The capsules were split open and the seeds transferred to 50-100 mL sterile distilled water to obtain 50-100 seeds per drop of seed suspension (OMA: Hollick, 2004) (pH 5.8). The seeds were sown onto the surface of Nylon netsterile of sterile OMA medium. The plates were inoculated the fungal inoculums (MLX102 and CLB111) taken from the actively growing hyphae edge 10 days after culturing on PDA. Uninoculated plates were used as a control. Ten replicates were maintained for each treatment and the whole experiment was repeated thrice. Petri dish plates were sealed with cling film and stored at the room conditions with day light (8:30 am to 5:30 pm) at 25 °C for 25 weeks. The cultures were examined after 2 weeks initially and further at monthly intervals under stereo microscope to assess germination and progress of protocorm and seedling development. Seed germination and seedling development were scored on 0–7 increment scale (modified from Stewart and Kane, 2007). Percentage of seed germination and protocorm development for each treatment was calculated using the relation: Number of seeds in each developmental stage /Total number of seeds with fully developed embryos*100.

Seed vigor determination and observation on mycelium infection

The optical microscope and electron microscope observation found that many fungus hypha infected into embryo cell and formed the 3D hypha net, and the embryo started differentiation. TTC measurement showed that the seed had high vitality.

No strain was found by re-separation from culture medium, no hypha was observed and very low vitality under control treatment (Figure 2a, b).

RESULTS

Fungal isolation and identification

Natural populations of *C. mastersii* possessed significant numbers of seedlings with endomycorrhizal colonization in their roots. Two *Rhizoctonia* sp. endophytic fungal isolates were recovered from their roots and one of them triggered symbiotic germination and supported enhanced growth of *C. mastersii* seedlings, as revealed in a preliminary examination. Molecular characterization of the isolate was obtained by PCR amplification of the ITS region using the primer pair ITS4 and ITS5 and sequencing of the 550 bp amplified product. This was followed by identification through comparing the sequence obtained with already available sequence in NCBI Genbank database using BLAST search tool (Wu *et al*., 2010).

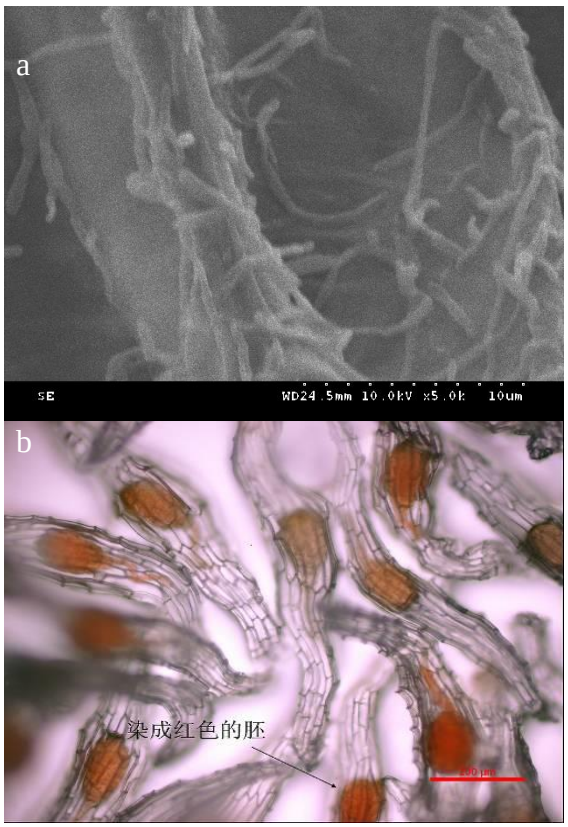

Figure 2. a, **b**: Hypha of CLB111 infected into the seeds and formed the 3D hypha ent (×5000), the seed became red with TTC stained seeds of *C. mastersii.*

Symbiotic seed germination

Seeds of *C. mastersii* germinated on OMA medium were previously inoculated with the symbiotic fungus CLB111. Early symptom of germination as swelling of embryos and rupture of seed coat occurred in 7 days and later they progressed to stage 2 in another 7 days. By 30 days, most of the germinated seeds were transformed into stage 3 with rhizoid formation and development of chlorophyll. Upper portion of the protocorm became bulged to form globular structure and leaf primordia were also developed during this stage. In another 30 days, 48% of the protocorms were progressed into stage 4 with the development of first leaf. However, majority of them died or did not grow further without symbiotic fungus CLB111 and MLX102. As the data was not promising for symbiotic seedling production, detailed data was not gathered (Figure 3a-h).

DISCUSSION

Propagation through symbiotic approaches is the most preferred option of threatened and endangered orchid species for their restoration into native habitats. Such methodology has already been applied in a series of terrestrial orchids and thus become a popular method for resto ration programs (Aggarwal and Zettler, 2010; Chutima *et al*., 2011; Sathiyadash *et al*., 2014). Even though extensive studies are not available, symbiotic propagation has also been proved or recommended as an effective method in a few epiphytic orchids (Khamchatra *et al*., 2016). Nevertheless, there is no study reporting symbiotic seed germination of *C. mastersii*, an endemic orchid of Himalayan region, China endangered due to habitat destruction. Thereby, this is the first report of *in vitro* symbiotic germination in this endangered terrestrial orchids.

Acknowledgements

This study was supported by the National Natural Science Foundation of China (31360198, 31260175, 31860208).

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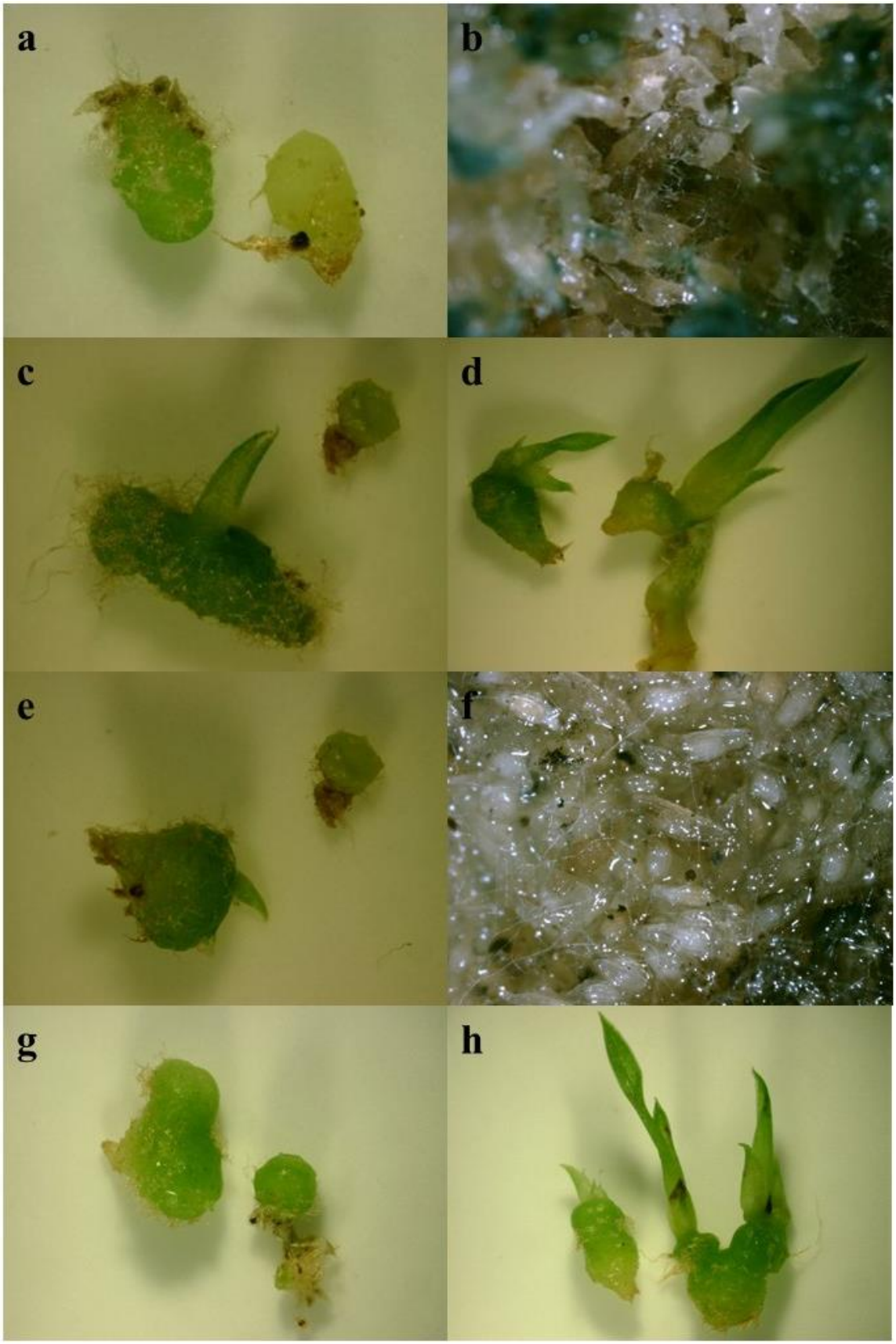

Figure 3. Germination of seeds of *C. mastersii*. **a**, **b**: Germination stages 1 of symbiotic seed with CLB111 and CK; **c**, **d** : germination stages 2 (left) and stages 3 (right) of symbiotic seed with CLB111 and CK; **e**, **f** : germination stages 1 of symbiotic seed with MLX102 and CK; **g**, **h**: germination stages 2 (left) and stages 3 (right) of symbiotic seed with MLX102 and CK.

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Nitrates affect orchid seed germination depending on orchid species and fungal symbiont

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Many orchid species recently disappeared from their sites without obvious reason and mature seeds of such species do not germinate in vitro, making its cultivation for scientific or rescue purposes impossible. We focused on nitrates as possible inhibitors of orchid seed germination. We tested whether seeds of various terrestrial orchids would be sensitive to nitrates at naturally occurring concentrations. We sowed seeds both symbiotically (*Ceratobasidium, Sebacina, Tulasnella*) and asymbiotically *in vitro*. The response differed markedly between taxa and was different in presence of fungal symbiont. In asymbiotic culture, *Pseudorchis albida* native to oligotrophic mountain meadows was extremely sensitive to nitrates while *Himantoglossum robertianum* and *Anacamptis laxiflora* which frequently occupy nutrient-rich biotopes like abandoned fields and eutrophic marshes, were nearly insensitive. The sensitivity generally correlated with trophy level of studied species. In symbiotic cultures, some fungi were able to induce germination even at concentrations, which were inhibitory in asymbiotic culture indicating that the fungi are able to modulate the nitrate effect on orchids.

Soil nitrate concentration has been increasing rapidly in last century. Therefore, nitrate deposition could be partially responsible for recent decrease in number of European orchid sites.

Suported by project LO1417 of Czech ministry of Education Youth and Sports.

Fungal diversity of seeds of *S. vomeracea* subsp. *laxiflora* and effects of these fungi on seed germination

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One of the world's largest plant family is Orchidaceae (Rasmussen, 1995). Many species are collected from nature for commercial purposes all over the world. Despite giving millions of seeds, germination in the nature is very difficult. Under natural conditions, germination depends on establishing a symbiotic relationship with a suitable fungus. In our research, it was aimed to determine fungal diversity and effects of these fungi to germination in the natural environment of *S. vomeracea* subsp. *laxiflora* seeds. The seeds were placed by dipping them near the plant roots. Seventy four fungi were isolated from the seed surface. In the morphological analysis of fungi, colony appearance, color, number of nuclei and growth rate were examined. The PCR protocol of the ITS1-5.8S-ITS2 gene region is described in Pascual *et al.* (2000). Phylogenetic trees were constructed using the algorithm of MEGA 4 (Tamura *et al.*, 2007) and Mr Bayes (Ronquist *et al.*, 2011). It was determined that one of the seed surface fungi belonged to *Tulasnella* genus and the other isolates belonged to *Fusarium, Pythium, Phoma, Alternaria, Aspergillus, Mortierella, Chaetomium, Arthrinium, Cunninghamella, Lecanicillium, Boeremia*. The fungi of the *Rhizoctonia* group obtained from the isolations promoted germination and non-*Rhizoctonia* fungi did not affect germination.

The research (Project No: 114Z218) was supported by The Scientific and Technological Research Council of Turkey (TUBITAK).

Symbiotic germination of the seeds and the seedling development of *Serapias vomeracea* subsp. *laxiflora*

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Serapias vomeracea subsp. *laxiflora* is one of the orchid species that collect tubers to make salep and all orchid species are under threat of extinction due to the collection of tubers and the destruction of natural areas in Turkey. For germination of orchid seeds in natural habitats, a suitable fungus is required. The suitable fungus/fungi are *Rhizoctonia*-like fungi that participate in mycorrhizal associations at the roots of adult orchid plants. For the production of orchids from seed, fungi stimulating germination should be determined. In this study, the fungi participating in mycorrhizal association in *Serapias vomeracea* subsp *laxiflora* roots were isolated and the effects of these fungi on seed germination in sterilized culture media have been determined. The isolations were done monthly for two years from the roots of three plants of *S. vomeracea* subsp. *laxiflora*. The fungi participating in mycorrhizal association with the roots were described by morphological and molecular methods and revealed that dominant fungus of mycorrhizal association was *Tulasnella.* In addition to, *Fusarium tricinctum, Aspergillus spelaeus* and *Talaromyces pinophilus* were isolated from the roots. All the fungi were used to symbiotic germination tests. *Tulasnella* spp. promoted the seed germination and seedling development, the other fungi did not.

The research (Project No: 114Z218) was supported by The Scientific and Technological Research Council of Turkey (TUBITAK).

Mycorrhizal fungi in *Platanthera chlorantha*

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Platanthera chlorantha is a common species occurring in *Quercus* forests in Turkey. Although this orchid is widespread, the collection of tubers and the destruction of forests are a serious threat to the future of its generation. In order to protect the orchids and improve the destruction, it is first necessary to isolate and identify the mycorrhizal fungi. For this purpose, mycorrhizal fungi in *Platanthera chlorantha* roots were isolated and identified by morphological and molecular methods. Eight isolates were obtained from the roots. Amplification of the rDNA-ITS region was done with the primers ITS4 /ITS5 (White *et al.*, 1990). Amplified products were sequenced by Macrogen Inc.(Korea). The sequences were aligned with CLUSTAL X (Thompson *et al.*, 1997). The identity of isolates was determined by making BLAST search. Phylogenetic trees were constructed using the algorithm of MEGA 4 (Tamura *et al.*, 2007) and Mr Bayes (Ronquist *et al.*, 2011). Two different *Rhizoctonia*-like fungi were identified according to anamorphic criteria. As a result of molecular analyzes, isolates M1 and M2, which were involved in mycorrhizal association, were associated with *Thanatephorus fusisporus* (HQ441575). *T. fusisporus* species were first identified in *Platanthera chlorantha* roots.

Trehalase in orchid mycorrhiza: colocalization with pelotons and gene multiplication

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Orchids rely on mycorrhiza, especially during the stage of heterotrophic protocorms. At this stage, fungi provide orchids with energy, carbon and other nutrients. It is unknown which compounds ensure the energy and carbon transfers.

To address the question, we selected *Dactylorhiza majalis* and *Ceratobasidium* sp. as the main model species. We focused on disaccharide trehalose which was hypothesized previously to play a role in carbon and energy transfers. Utilization of this saccharide depends on trehalase action and we therefore sought to localize trehalase in mycorrhizal tissues. We developed a histochemical trehalase localization method. Our results show, that trehalase activity tightly colocalizes with mycorrhizal structures. The same pattern was observed in completely heterotrophic protocorms as well as in roots of adult plants. In addition to this, we searched trehalase genes in embryophyta and reconstructed their phylogeny. We identified trehalase gene multiplication in *Dactylorhiza majalis* possessing five trehalase paralogs. Strikingly, no such multiplication occurred in other orchid subfamilies.

The results suggest that fungal trehalose can be hydrolysed by trehalase directly in mycorrhizal tissue, which could be reflected by trehalase gene multiplication.

The work was supported by Ministry of Education, Youth and Sports of the Czech Republic (project NPU1: LO1417).

Mycorhizal diversity of epiphytic orchids in a hyperdiverse tropical forest: insights on temporal and life stage changes

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Few studies have examined the diversity of fungal communities of epiphytic orchids from hyperdiverse tropical forests and its temporal dynamics. This study aims to describe the fungal diversity associated with three tropical, epiphytic orchid species from a Costa Rican hyperdiverse forest across a three year-period at the juvenile and adult stages, by applying standard Sanger sequencing methods.

Results show that fungi belong to orders Cantharellales (most common clade), Atractiellales and Trechisporales. This is the first report of Trechisporales in photosynthetic orchids. Members of the Cantharellales and Atractiellales were found in *D. fragrantissima* and *O. klotzschianum*, while *E. odontochilum* had mycobionts only from Cantharellales and Trechisporales. Mycorrhizal diversity was higher in the common *O. klotzschianum* (20 OTUs recognized), while the rare *D. fragrantissima* and *E. odontochilum* hosted 9 and 7 OTUs, respectively. These results suggest a broader mutualistic interaction in *O. klotzschianum*, which may confer more opportunities for establishment, and narrow associations in the scarce species, which may favor growth and competitive dominance. Within each species, less than three mycobionts were shared between years, revealing high unprecedented dynamism on the relationships between orchids and their mycorrhizal composition through time.

Why don't orchid pollinators go extinct? The persistence of the costly coevolutionary relationship between the sexually deceptive *Cryptostylis* orchids and their duped pollinator

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The sexually deceptive Australian and New Zealand *Cryptostylis* orchids are extreme deceivers: causing their wasp pollinator, *Lissopimpla excelsa* to ejaculate and waste limited sperm. Despite exerting great costs upon their pollinators, these orchids achieve outstanding pollination rates. Why, then, don't their pollinators go extinct?

We propose that costs are buffered by counter-adaptations, and a new concept, species-level 'resilience' traits. Field experiments at sites with (n=3) and without (n=3) natural populations of orchids in Sydney, Australia revealed evidence for counter-adaptations. We found evidence that male pollinators in sympatry with orchids spend less time mating with orchids; are less likely to waste sperm; and have more sperm and longer antennae than those that are not.

We produced a mathematical model to investigate the role of resilience traits. These traits allow a species to avoid extinction: reducing an individual's ability to escape exploitation via counter-adaptations whilst maintaining exploiter fitness. For sexual deception by *Cryptostylis* orchids, Haplodiploidy could act as a resilience trait. Unmated Haplodiploid pollinator females deprived of sperm by orchids can still produce offspring, albeit all sons. An overabundance of sons would further enhance orchid pollination rates and fitness, while providing enough males to maintain pollinator populations. Our model found that when exposed to the same costs, haplodiploid wasps (e.g *L. excelsa*) persist over evolutionary time while diploid wasps become extinct.

New findings on the pollination of European orchids

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Attracting potential pollinators is of vital importance for orchids, for most orchids depend on insects for the transport of the genetic material, the pollinia. Observations showed that some insect species can pollinate a wide range of orchid species, whereas other orchids are adapted to a specific pollinator. *Empis* species proved to be pollinators of *Neotinea ustulata*, *Neottia ovata*, *Dactylorhiza fuchsii*, *Gymnadenia conopsea* and *Epipactis palustris*. Various *Bombus* species are the main pollinators of *Epipogium aphyllum*. We found Crambidae and Diptera to be the main pollinators of *Gymnadenia rhellicani*.

Liparis stricklandiana (Orchidaceae, Liparidinae) – study of flower structures in the context of the pollination processes

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In our report we present results of an investigation concerning the pollination pathways in *Liparis stricklandiana* (Orchidaceae, Liparidinae). During conducted study we have observed the signs of secretion at the lip surface. This fact has led us to investigate flower samples by means of both scanning (SEM) and transmission electron microscope (TEM). As the result of performed analysis, we have confirmed for the first time secretion of floral liquid attractant at the species flower elements, especially at the lip. Additionally, we performed the analysis of morphology of flower elements and pollination functions.

What is more, observation made upon living plants, let confirm the facultative process of self-pollination for *Liparis stricklandiana*, which occur at the end of anthesis. As further research revealed, this kind of phenomenon has never been described before for this species.

Subtribes Malaxidinae and Liparidinae (Orchidaceae, Malaxideae) – taxonomic divergency as effect of pollination isolation

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Abstract – Fundamental for the process of divergence within tribe Malaxideae (especially between subtribes Malaxidinae and Liparidinae) are: different landing places for visiting insects on flower, insect visiting position in relation to the gynostemium. Consequence of the differences is another place of pollinia deposition on a pollinator body. It preventing effective pollination of flowers of one subtribe by insects effectively pollinating the flowers of the other subtribe.

Key words: Liparidinae, Malaxideae, Malaxidinae, Orchidales, taxonomic divergency, pollination isolation

INTRODUCTION

Tribe Malaxideae with its over 1700 taxa (including synonyms) is undergoing an extremely active phase of speciation as is evidenced by e.g. a high degree of morphological variability (inclusive of ontogenetic variation) (Margońska *et al*., 2013).

Crucial in the process of divergence of tribe Malaxideae (subtribes Malaxidinae and Liparidinae) are differences in the landing places for insects on flower and the visiting position of their pollinators in relation to the gynostemium. All this results in a different place of deposition of pollinia on a pollinator body. The increase in the specialization of the floral structures form therefore promotes reproductive isolation between representatives of the subtribes.

MATERIALS AND METHODS

The object of the studies was representatives of both subtribes Malaxidinae and Liparidinae (Malaxideae). Observation of pollination strategy, anthesis of the orchids etc. were conducted in natural stands (*in situ*) and in glasshouses conditions (*ex situ*). Researches were performed on the basis of preserved (dried, conserved in Copenhagen mixture etc.) and live materials. Flowers structures examination was carried out with using standard morphological and anatomical observation (light stereoscopic microscope), scanning (SEM) and transmission electron microscopy (TEM) analysis, also cytochemical tests (CYTO). Olfactory and secretory emissions were also subjected to chemical analysis.

RESULTS

Subtribe **Malaxidinae (**"*Malaxeae*" Benth. & Hook.f., Gen. Pl. 3: 463, 465. 1883.) comprises species with flowers 360° resupinate, lip directed up (except only *Micr. monophyllos* subsp. *brachypoda*, *Micr. muscifera* (Lindl.) subsp. *stelostachya* (Tang&Wang) Marg., *Micr. yunnanensis* and *Tamayorkis*). Lip is parallel to gynostemium, with a distinctly reduced hypochile, while the epichile can be 3-lobed (middle and 2 lateral lobes) or 1-lobed. Epichile contains differently formed and ornamented 2-3 chambered (never globular) concavity. Gynostemium is column short up to 2-3 times as long as the anther. Anther is erect, parallel to the column and stigma (except only the mountain genus *Tamayorkis*), its locules opening ventrally or apically (never laterally). Stigma opening apically and situated inside a deep pocket. All mentioned morphological characters of Malaxidinae flowers make gynostemium and/or partly dorsal sepal the place of pollinating insects landing (Figure 1).

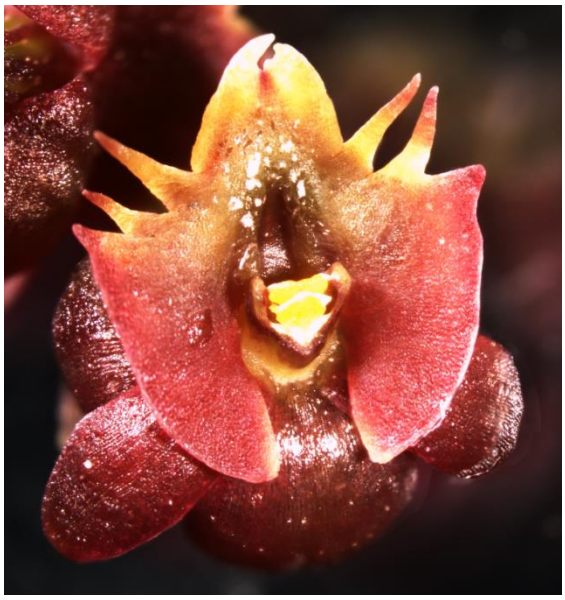

Figure 1. *Crepidium hoi*, flower: pollinia will deposited dorsally at pollinator body.

Subtribe **Liparidinae (**"*Liparidae*" Lindl. ex Miq., Fl. Ind. Bat., 3: 618, 621. 1855. *emend* Margońska *et al.* 2012 (2013)) flowers are 180° resupinate with lip di-rected down (except of hanging down epiphytes such as *Alatiliparis*, *Platystyliparis* and *Crossoglossa*). Lip is distinctly divided on the well-developed hypochile and epichile. Nectary are present usually in a form of smooth area around the lip base and/or its basal callus/calli/lamellae/ globular structure (*Alatiliparis*, *Disticholiparis*, *Platystyliparis*) if they exist. Well visible is usually darker coloured and shiny stripe (central thickening, sometimes called as pseudonectary) reaching from the lip base to distal part of lip epichile. Gynostemium column is elongated, from 2-3 times or more as long as the anther (except *Crossoglossa* and *Crossoliparis* where the gynostemium length is similar to the anther length). Anther is always orthogonal to the column, staminodes and stigma and its locules opening ventrally or laterally (never apically). Stigma opening ventrally (inside a deep concavity not a pocket). The most exposed element of the flower is here epichile of the lip which is landing place for pollinating insect (Figure 2).

Additionally within the Malaxideae the nectar, if any, is secreted in very limited amounts, which probably forces the insects to visit many flowers before they satisfy their hunger – constituting an undeniable advantage for the orchids.

Figure 2. *Liparis* (*Stichorkis*) *crenulata*, flower: pollinia will deposited ventrall at pollinator body.

DISCUSSION AND CONCLUSION

The concavity of Malaxidinae seems to lure insect by imitating the secrecy of attractants. Its epidermis for example is smooth and shiny, simulating the presence of a sticky liquid. Little amount of very minute droplets of secrecy was observed only above of the concavity border. The concavity and its surrounding is available for visiting insects only after landing on just gynostemium and/or partly on dorsal sepal. Reaching for the concavity, the insect from above must force itself between the concavity and the gynostemium, its position exactly coinciding with the apically opening pocket-like stigma. In this way the insect can deposit on the stigma the pollinia if have brought from a previously visited flower. When the insect withdraws, it unhooks the anther, which, upon bending, attaches the pollinia to the underside of the animal.

Within Liparidinae flowers the secretory area, if it presence was confirmed, is located below the lip basal callus/calli/lamellae/ globule. The structure of the epidermis cells of the lip's suggests possibility of the dripping/ exudation of the secretions towards the central part of the lip, than in the direction of central thickening and finally down the lip. Liparidinae secretory area is reachable for visiting insects after landing on epichil and following along the central thickening as nectar-guide. The insect must force itself

between the canaliculated hypochile and the gynostemium, its position exactly coinciding with the ventrally opening concaved stigma. In this way the insect can deposit on the stigma the pollinia if have brought from a another flower. When the insect withdraws, it unhooks the anther, which, upon bending, attaches the pollinia to the back of the animal.

Therefore, the probability of crossed effective pollination between representatives of both subtribes becomes impossible.

Acknowledgments

I am grateful to curators and staffs of Herbaria, Libraries for making accessible a taxonomic, comparison materials and/or their hospitality and assistance during my personal visits. I am especially indebted to M.Sc. Karolina Bednarska for her assistance in digitalisation of scientific data.

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Differences in spur length of *Habenaria tridactylites* on the Canary Islands suggest an evolutionary arms race with pollinators

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Virtually nothing is known about the pollination of *Habenaria tridactylites*, an endemic orchid of the Canary Islands. This species grows in a zone influenced by moisture providing trade winds. The entirely green, widely open flowers have a long spur containing nectar. Most *Habenaria* species are moth-pollinated. In this study, we investigated: 1. by which moth species this orchid is pollinated and 2. whether there is a relationship between mean spur length and tongue length of local flowers and pollinators and 3. if there is a relationship between mean spur length and the age of the island, altitude, latitude, and surrounding vegetation. Our study showed that *H. tridactylites* is pollinated by both small and larger moth species. Analysis of data collected at various sites indicates that there seems to be a correlation between the age of the islands and the mean spur length of local populations of *H. tridactylites*.

Pollination strategy of a food-deceptive orchid: a community approach

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Traunsteinera globosa is a food-deceptive orchid assumed to mimic a guild of plants with locally differing model species. Its major pollinators are generalist syrphid and empidid flies. In previous studies, putative model plants were mainly selected by visual similarities to the orchid judged by the human eye, and quantitative analysis of visual and olfactory floral cues restricted to these species. Similarly, the pollinator spectrum was only investigated for *T. globosa* and these models. It remains unknown whether this orchid shares pollinators also with other co-flowering plants and if so, how similar their floral traits are to the orchid and the putative models. We analysed visual and olfactory flower cues of an alpine plant community containing *T. globosa*, observed the pollinator network, tested the importance of scent for pollinator behaviour in an experiment, and identified physiologically active scent compounds. Results show that besides the putative models another eight co-flowering plants shared pollinators with the orchid. Six of them were fly-blue as the orchid, two were fly-purple. All co-visited plants shared a number of physiologically active scent compounds with the orchid, but the overall scent blends differed among the species. The behavioural experiment showed that pollinating flies use the orchid's scent as an attractive cue. These results point to a more generalist pollination strategy of *T. globosa*, involving not only the putative models, but also other co-flowering plants.

The orchid genomic toolkit

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The application of genomic studies to orchids enables increasingly detailed discoveries of the evolution of both species and organs. We discuss different evolutionary questions that can be addressed using such approaches, and indicate optimal sequencing and data-handling solutions for each case. With this article we hope to promote the diversity of genomic research capitalizing the fascinating natural history of orchids that can now even be completed within the timeframe of a single PhD project.

Why sequence orchid genomes?

Sequencing methods keep diversifying and their costs continue to drop (van Dijk *et al*., 2014). This opens up new perspectives for orchid studies previously hampered by the lack of well-established genetic model species. Nowadays, the **genomes** (see glossary in Box 1) of organisms with no pre-existing genetic resources can be mined quite easily (Matz, 2017). And if optimal approaches are followed, new insights in orchid phylogenomics or gene regulation of for instance floral characters underlying adaptations against pollinators can be obtained in the timeframe corresponding with a single PhD project.

Orchid reference data currently available

Genomic studies are all organized in a similar way: anonymous **reads** are sequenced from an organism, and these reads are subsequently matched with a reference, either a genome or **transcriptome**. The reads can be short and the reference sequences do not need to be very accurate; they should only be sufficiently correct to allow unambiguous matching with reads produced from the study subject. Currently, genome sequences are available for a total of eighteen orchid species belonging to all five subfamilies of which further details are listed under Resources.

Previous orchid phylogenetic studies required labor-intensive marker development coupled with polymerase chain reactions of a single **locus** and **first generation DNA sequencing**. These techniques brought many important first insights in orchid evolution such as the most basal phylogenetic position of the Apostasioideae, followed by Vanilloideae and Cypripedioideae and a more derived position of the Orchidoideae and Epidendroideae subfamilies (Chase *et al*., 2003), the first estimate of the age of origin of the family (Ramirez *et al*., 2007) and the first set of developmental genes responsible for perianth formation as described in the orchid code model (Mondragon-Palomino and Theissen, 2011) that can be considered an expansion of the ABCDE model (Coen and Meyerowitz, 1991) and floral quartet model (Theissen and Saedler, 2001). **Next generation DNA sequencing** techniques, enabling high-throughput parallel sequencing of short DNA molecules of multiple samples, added new insights in the most important drivers of orchid evolution such as epiphytism, evolution of pollinia, and CAM photosynthesis (Givnish *et al*., 2015).

Despite major breakthroughs, though, several nodes in orchid phylogenies remained unresolved with first and next generation sequencing techniques, due to processes such as incomplete lineage sorting, hybridization, or gene duplication, that cause reticulate patterns among the relationships of species. Such cases can now be resolved with **Anchored Hybrid Enrichment** (Figure 1), an innovative next generation sequencing technique, producing data from hundreds of loci of potentially hundreds of individuals for both deep and shallow phylogenetic analyses in a single run (Lemmon *et al*., 2017). For this technique, (i) **probes** are first designed for target enrichment of ca. 500 loci in highly conserved regions in

Box 1. Glossary

Anchored Hybrid Enrichment: a method using conserved probes to recover a large number of innovative phylogenetic markers from chloroplast, mitochondrial and nuclear genomes
Annotation: the process of identifying the locations of the coding regions in a genome and determining what those regions do
Assembly: the process in which short DNA or RNA fragments are merged into longer fragments in an attempt to reconstruct the original sequence
Bioinformatics: the application of computational biology to handle the rapidly growing repository of genomic data
Coverage: the number of times that a given nucleotide in a sequence is sequenced
Datamonkey: a public server for analysis of sequence data
***de novo*:** lacking a reference
First generation sequencing: methods such as the Sanger technique for sequencing short individual DNA and RNA molecules
Genome: the complete set of genetic material present in a cell or organism
GitHub: an open source platform for software development
Locus: a fixed position on a chromosome where for instance a coding region is situated
Next generation sequencing: techniques such as Illumina or Ion Torrent for high-throughput parallel sequencing of short DNA or RNA molecules from multiple samples in a single run
Probe: a small fragment of DNA or RNA binding to a sequence that is complementary to its own
Read: a sequence of base pairs corresponding to a single DNA or RNA fragment
Script: the most basic part of a bioinformatics pipeline and written in a special programming language
Third generation sequencing: techniques such as Single Molecule Real Time (SMRT) sequencing by PacBio or nanopore sequencing by Oxford Nanopore Technologies for sequencing long individual DNA and RNA molecules
Transcriptome: the expressed part of a genome

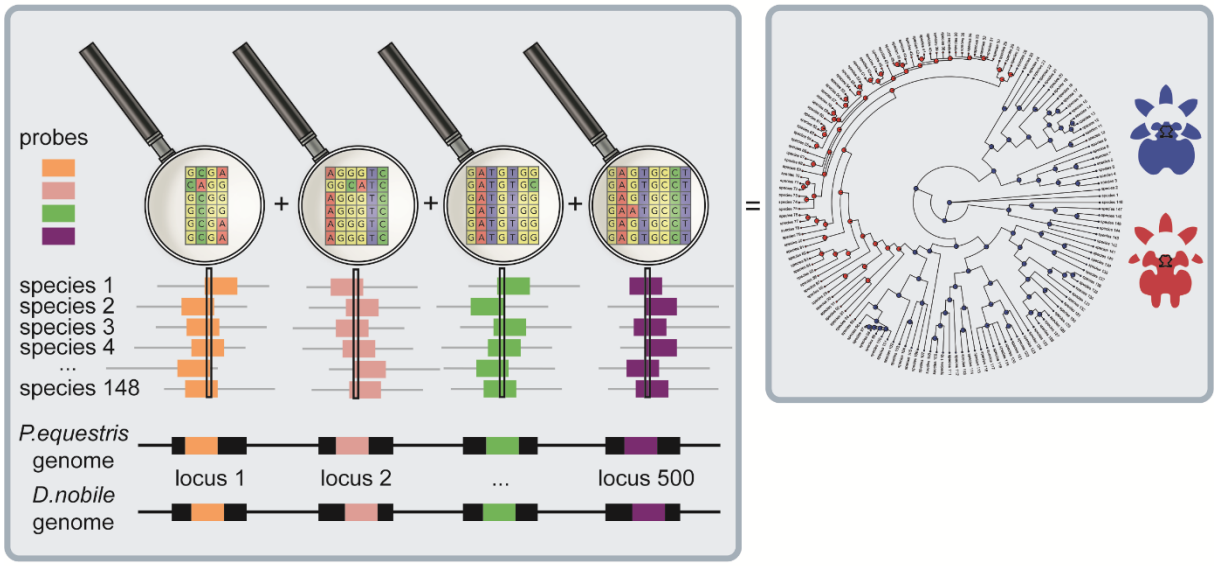

Figure 1. Graphical summary of the Anchored Hybrid Enrichment method. Color legends: black = reference genomes; orange, pink, green and purple = probes; grey = reads obtained from regions captured with probes in the genomes of the study species; blue = flowers with similar shaped sepals; red = flowers with differently shaped sepals. Illustration by Bas Blankevoort.

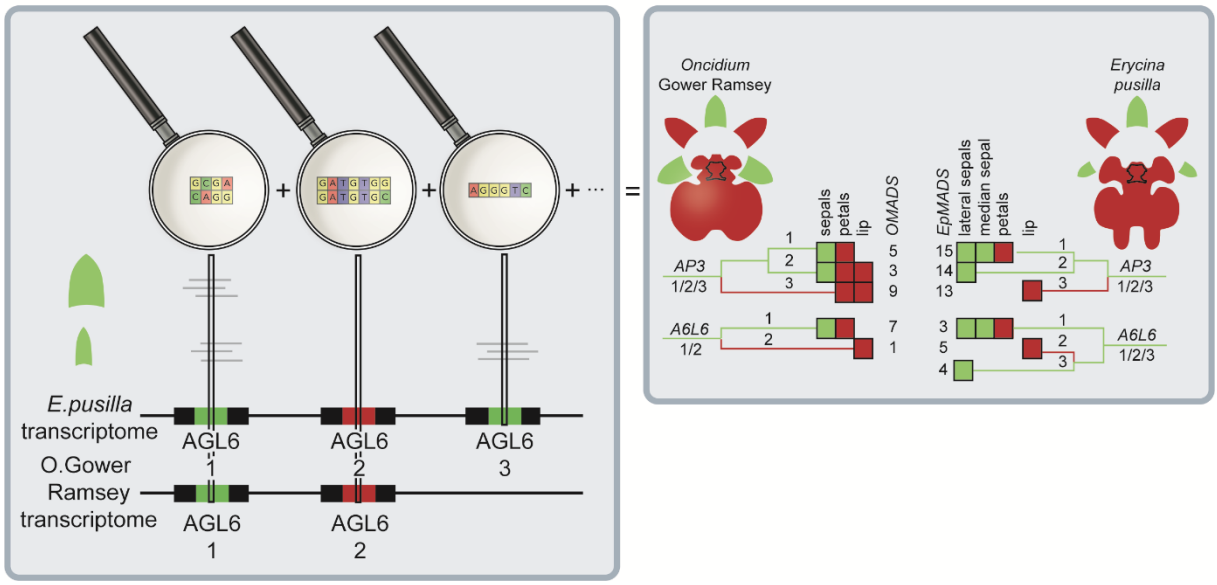

Figure 2. Graphical summary of transcriptome analyses. Color legend: black = transcriptomes; green and red = *AGL6* locus of which *E. pusilla* possesses three copies in its genome but *O.* Gower Ramsey only two; grey = reads obtained from *AGL6* gene copies expressed in differently shaped sepals. Illustration by Bas Blankevoort.

the genomes of reference species for which such data are available like for instance *Phalaenopsis equestris* and *Dendrobium nobile*; (ii) subsequently, enrichment of the genomes of the study species with these probes, called anchored enrichment, capable of recovering a large number of unlinked loci in either the chloroplast, mitochondrial or nuclear genome, is carried out; (iii) loci sequenced from the study species are then processed for **annotation** using an automated **script** in a **bioinformatics** pipeline of which further details are listed under Resources and (iv) used to reconstruct separate gene lineage trees, which can be used to infer a species tree. Due to the large number of gene lineage trees produced from both biparentally inherited nuclear loci and maternally inherited chloroplast and mitochondrial ones, hybridization patterns can be detected and resolved. This technique can therefore increase resolution of shallow orchid clades that remain unresolved with other sequencing techniques (Bogarín *et al*., in press), even when limited or no genomic resources are available for the target species themselves. Another added value of this technique is that DNA extracted from herbarium specimens can be processed as well (Hart *et al*., 2016), increasing sampling options for endangered or rare orchid species. Resolving the resolution of shallow orchid clades opens up the possibility of tracing character state evolution so that evolutionary informative characters can be separated from repeatedly evolving ones. The next challenge is to recover the genetic basis of evolutionary informative characters. This can be done with a transcriptome analysis (Figure 2), which is a cost-efficient alternative to whole-genome sequencing for gene expression studies. Ideally the transcriptome should be collected from the organ of which the genetic basis is sought, preferably harvested from buds and mature tissue, to find out which genes are involved in the early stages of development and which ones in the later stages. The standard way to generate a ***de novo*** transcriptome is to first produce RNA sequences with high **coverage** and then apply (i) data filtering to remove for instance contaminating sequences from endophytic bacteria and fungi, (ii) **assembly**, (iii) quality assessment and (iv) annotation tools using dedicated bioinformatics pipelines, of which further details are listed under Resources.

Massive parallel sequencing of short RNA molecules added important additional insights in the genetic basis of the orchid sepals, petals and lip, as described in the Perianth Code model (Hsu *et al*., 2015; Gravendeel and Dirks-Mulder, 2015) and Oncidiinae model (Dirks-Mulder *et al*., 2017). It also provided the first glimpses of the genetic basis of other orchid organs in the third and fourth floral whorls such as the stamen and stelidia (Dirks-Mulder *et al*., 2017). Ongoing developments in **third generation sequencing** technologies will soon enable retrieval of even higher quality transcriptomes by sequencing longer reads that are expected to ultimately encompass full-length transcripts (Hoang *et al.*, 2017).

Concluding remarks

In the past decade, several new genomic techniques were developed, accompanied by innovative bioinformatics tools. These methods now enable addressing fundamental evolutionary questions for any orchid species within a relatively short timeframe. The last remaining recalcitrant shallow nodes in orchid phylogenies can be resolved relatively fast when applying a team approach as recommended in Box 2. These resolved phylogenies could then be used for tracing character evolution to unravel the full genetic basis of the highly specialized organs that make orchids such fascinating subjects for evolutionary studies.

Box 2. Best Practices for Orchid Genomics

1. Apply different data-filtering settings to ensure robust results and report the exact settings used
2. Share new sequencing data, scripts and other bioinformatics tools with the orchid community in open-access repositories such as **GitHub**
3. Use partially overlapping probes in Anchored Hybrid Enrichment projects so that separate studies can be combined
4. Work on public servers such as **Datamonkey** for analyses that demand a lot of computational power

Resources

http://orchidstra2.abrc.sinica.edu.tw
https://github.com/naturalis/orchids
https://github.com/naturalis/orchid-transcriptome-pipeline/

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Biotic stress is driving divergence and sequence evolution in *Dactylorhiza fuchsii* and *D. incarnata*, a pair of species with distinct ecological preferences

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Although Orchidaceae are the largest family in the angiosperms, little is known about the genetic basis of this significant biological variation. We have taken advantage of the methods used to sequence genomes to investigate the genetic divergence between two food-deceptive European terrestrial orchids, *Dactylorhiza incarnata* and *D. fuchsii*, which we integrate with their distinct ecologies, which we also document. Clear lineage-specific adaptive features are identified, in particular elements of biotic defense (relating to attacks by viruses and bacteria), in agreement with the Red Queen hypothesis (which proposes that organisms must constantly adapt, evolve, and proliferate not merely to gain reproductive advantage, but also simply to survive while pitted against ever-evolving opposing organisms in an ever-changing environment). We show the two *Dactylorhiza* species inhabit distinct niches; they differ significantly with regard to soil acidity and tree cover, but also with respect to temperature evenness over the year and precipitation of the driest month. If maintained over generations, such deviating ecological preferences likely have triggered distinct selection that in combination to specific demographic histories have moulded different genomic landscapes in these two species. The current prevalence of *D. incarnata* within small, localized populations over a highly fragmented distribution is corroborated here with considerable levels of inbreeding in this taxon. In contrast, *D. fuchsii* currently grows in larger, more diffuse populations and exhibits higher levels of heterozygosity and greater genetic diversity. When the two species are grown in a common garden intermediate between the two, the major features under selection relate to adaptation/acclimation to abiotic conditions (primarily water relations). These genomic techniques are not very expensive and offer for the first time the possibility to investigate which factors in the life history of orchids are driving their evolution, making it possible for scientists to begin the process of developing a model of how orchid diversity has evolved.

Is genetic technology approaching the limit of its ability to help us understand the systematic biology of orchids? The broader implications of a case-study in *Epipactis*

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By the beginning of the 21st Century, genetics appeared to show every promise of revolutionizing every aspect of systematic biology. Molecular phylogenetics would result in much more stable classifications, while population genetics would objectively circumscribe species. DNA fingerprinting techniques would, once miniaturised, allow DNA-based identification of plant materials in the field. Genetic modification techniques would allow a much deeper understanding of plant development, telling us precisely what makes an orchid an orchid. 18 years later, we can see that progress toward this 'brave new world' has been slower than predicted, and that technological expertise has expanded more rapidly than the knowledge that it was expected to generate. Recent molecular phylogenies contain orders of magnitude more information than their predecessors but offer little more confidence in their accuracy. Species continue to be described through authoritarian pronouncement, in the absence of any underpinning science. Hand-held DNA sequencers for field use remain in the realm of science fiction, perhaps because the enormous size of the potential market has not yet been realised by developers. And translating knowledge of the genetic code into genuine understanding of mature morphology has been confounded by the spectacular array of epigenetic processes that should now be the main focus of biological research but as yet are not. In summary, progress is still being made, but we now have a far more realistic view of what can and cannot be achieved.

Genetic divergence and ecotype formation in *Epipactis* - Implications for conservation

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Ecological speciation is the process by which one species diverges into two distinct phylogenetic lineages that gradually become reproductively isolated from each other after they have colonized a new habitat. Because ecological speciation typically occurs across a continuum of time and several intermediate stages, often called ecological races or ecotypes, can be discerned during the speciation process, it may result in a complex of taxa among which species limits are difficult to define. A typical example of a species group among which species limits are difficult to define is the genus *Epipactis*. It contains a complex of autogamous and non-autogamous taxa that may have arisen after the colonization of new habitats, followed by rapid adaptation and evolutionary changes in key traits that allow establishment and survival in these newly colonized habitats. However, the taxonomic status of these species is problematic and different authors have treated the taxonomy of *Epipactis* in different ways, some recognizing the different taxa as distinct species, others considering them only as minor intraspecific variants or ecological races. Here we present the results of genomic, meta-genomic and morphological analyses aimed at investigating the taxonomic status of coastal dune populations of the widespread terrestrial orchid *Epipactis helleborine*. Investigations of the mycorrhizal fungi associating with coastal dune populations and typical forest populations has shown that they associate with significantly different fungal communities. Crossing experiments show that both taxa easily cross and produce viable seeds. However, germination of seeds of dune populations in forest habitat and vice versa was always lower than that of seeds of coastal populations in dune habitats or of forest populations in forest habitat, leading to strong reproductive isolation as a result of immigrant inviability. Genomic analyses using SNP markers further revealed that coastal dune populations diverged only about 50 generations ago from inland populations, went through a significant bottleneck and were most likely the result of a single colonization event. Current levels of population genetic diversity in 27 populations along the Dutch and French coast were low and not related to population size or spatial isolation. The sampled dune populations also showed very little genetic differentiation and no apparent spatial genetic structure was observed. Overall, these results are consistent with a process of genetic divergence after a single, very recent colonization event followed by extensive gene flow among populations. From a taxonomic point of view, coastal dune populations of *E. helleborine* should not be treated as a separate species, but rather as an ecotype.

Multi-omics approaches provide insights into fungal-plant interactions in the model system *Serapias vomeracea* - *Tulasnella calospora*

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Orchids are highly dependent on their mycorrhizal fungal partners for supply of organic carbon and other nutrients, especially during seed germination and early development. We have used genomics as well as untargeted transcriptomics and metabolomics to investigate plant-fungus interactions in the symbiotic association formed between the terrestrial orchid *Serapias vomeracea* and *Tulasnella calospora* (Basidiomycota, Cantharellales). Transcriptomic profiling was instrumental to get insights on the symbiosis-upregulated plant and fungal genes that may play a role in the orchid mycorrhizal interaction. Among them, we could identify genes involved in nitrogen uptake, which allowed us to reconstruct the possible pathways of nitrogen transfer from the fungus to the mycorrhizal protocorms.

Metabolomic profiling was used for the first time in orchid mycorrhiza to investigate metabolic changes that occur in *S. vomeracea* and *T. calospora* during interactions. Even though data are preliminary, the availability of the *T. calospora* genome (Kohler *et al.*, 2015), and the possibility to compare metabolomic and transcriptomic data for both plant and fungus, open new perspectives for a comprehensive understanding of the pathways modulated by the symbiosis.

Note: RNA sequencing has been carried out at the US Department of Energy (DOE) Joint Genome Institute (contract number DE-AC02-05CH11231) within the framework of the Community Sequencing Project #978 "The Mycorrhizal Genomics Initiative: Exploring the Symbiotic Transcriptomes", coordinated by F. Martin (INRA-Nancy).

Genome size and phylogenetics of subfamily Vanilloideae inferred from NextGen anchored phylogenomics

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Phylogenetic relationships among genera of tribe Vanilleae continue to remain elusive. One of the possible reasons is that five of the nine genera of the tribe are mycoheterotrophs. These orchids are poorly represented in earlier molecular studies that relied heavily on plastid loci. Interestingly, even the relationships among some photosynthetic genera remain unresolved (e.g., the relationship between South American *Epistephium* and the clade of New Caledonian endemic genera *Clematepistephium* + *Eriaxis*). We have inferred the phylogeny of tribe Vanilleae through the use of Anchored Phylogenomics that targets ca. 500 low copy nuclear genes via Next Generation DNA Sequencing. Furthermore, we are able to recover the plastome sequence from most of our sample, and compare the trees. Our results are different from any previous study in that they show *Epistephium* forming a clade with *Clematepistephium* + *Eriaxis*, as well as *Lecanorchis*. Challenges were met in sequencing members of tribe Pogonieae for outgroup analysis because of the enormous genome size exhibited by these orchids (the largest in the family). As such, we embarked on a study also to estimate genome size for most genera of Vanilloideae. Not only does the subfamily have the largest known orchid genome, but it also contains the largest diploid chromosome number reported for Orchidaceae: *Epistephium lucidum* (2n= ca. 170). Our data shows that there is at least an 18-fold range of genome size in Vanilloideae, from 2.985 pg for *Eriaxis rigida* to 55.4 pg in *Pogonia ophioglossoides*.

Time-dependent diversification under high species turnover shapes species richness disparities among tropical rainforest lineages of *Bulbopyhllum* (Orchidaceae) on a global scale

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Tropical rainforests (TRFs) harbour almost half of the world's vascular plant species diversity while covering only about 6–7% of land. However, why species richness varies among the Earth's major TRF realms [i.e. tropical America: *c.* 93,500 spp.; Asia-Pacific region: 61,700; mainland Africa/Madagascar: 20,000] remains poorly understood. Here we investigate the evolutionary processes shaping continental species richness disparities of the pantropical, epiphytic and mostly TRF-dwelling orchid genus *Bulbophyllum* (*c.* 1962 spp. in total; Asia: *c.* 1570 spp.; Madagascar: 212; Africa: 84; Neotropics: 96) using diversification analyses based on a time-calibrated molecular phylogeny (containing *c.* 13.25% of extant species), coupled with ecological niche modelling (ENM) of geographic distributions under current and past (last glacial maximum) conditions. Our results suggest that the variation of species richness among regional TRF lineages of *Bulbophyllum* is best explained by a time-for-speciation effect rather than differences in net diversification rates or diversity-dependent diversification due to current or past spatial-bioclimatic limits. Why these lineages diversify under high rates of speciation and extinction (i.e. high species turnover) deserves further study but might relate to various intrinsic features commonly invoked to foster rapid population turnover in tropical orchids (e.g., epiphytism, specialization on individual pollinators and mycorrhizal fungi, resource-limited reproduction).

DNA barcoding of the *Epipactis* taxa native in Greece

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The genus *Epipactis* Zinn. constitutes one of the most difficult taxonomically genera of the family Orchidaceae, both in Greece and in the rest of Europe. This is mainly due to the breeding system and the remarkable morphological variability of its taxa, which, during the last 30 years, led to the description of many new taxonomic entities. In the present work, 22 taxa of the genus *Epipactis* native in Greece were studied using the DNA barcoding regions *rbc*L, *mat*K and ITS-2. *Epipactis pinovica*, a species recently described, was also included. One to five specimens from each taxon were used in the analyses. The *rbc*L sequence was highly conserved, whereas the *mat*K and ITS-2 sequences showed a significant number of SNPs. Based on those SNPs, 12 taxa (including *E. pinovica*) were discriminated, while the rest formed groups of 2-4 species showing the same polymorphism. These results reflect the taxonomic complexity of the genus and the possible misclassification of some morphological variants as distinct species, whilst the respective cases are presented and discussed.

Pleurothallidinae – a hyperdiverse subtribe with hyperdiverse genomes

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Although endoreplication of nuclear DNA is an important part of the cell differentiation process in many plant groups, progressively partial endoreplication (PPE) is a specific feature for about one third of orchid species from various phylogenetic groups. One of such is the understudied subtribe Pleurothallidinae, comprising more than 5000 morphologically highly diverse species in 20 to 100 genera native to tropical America. Pleurothallidinae represents an ideal model system to deepen our knowledge of PPE, and to investigate the orchid genome structure and its evolution. Therefore we employed Hyb-Seq sequencing, flow cytometry and karyology to get insight into phylogenetic relationships within Pleurothallidinae, nuclear genome sizes and endoreplication patterns, and chromosome numbers and rearrangements, respectively. Our preliminary phylogeny mostly supports the current taxonomic division. Nuclear genome size (1C-value) ranges from 0.22 to 5.41 pg and chromosome numbers span from 12 to more than 60, however, no direct link between the both traits was found. Approximately one half of the investigated species exhibit PPE, and the minimum proportion of replicated genome is about 19%. Our results indicate that the hyperdiverse morphological variation in Pleurothallidinae might stem from their extremely diverse genomes and a high diversification rate.

Evaluating the performance of anchored hybrid enrichment generated nuclear, plastid and mitochondrial markers in the species-rich genus *Lepanthes* (Orchidaceae: Pleurothallidinae)

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Most of the phylogenetic studies in Orchidaceae are based on inferences obtained from the sequencing of few molecular markers. However, these inferences usually do not solve recalcitrant nodes in phylogenies. To tackle this problem, new sequencing techniques such as Anchored Hybrid Enrichment (AHE) allow the obtaining of ~ 500 orthologous loci, thus increasing the amount of information analyzed. This technique has been evaluated in several plant groups, including Arecaceae, Fabaceae, Lamiaceae, Oxalidaceae, Pinaceae, Proteaceae, Serraceniaceae and Zingiberales yielding better resolution to recalcitrant nodes in phylogenies. However, these phylogenetic studies on multi-locus datasets found high levels of discordance and conflicting topologies due to biological phenomena such as hybridization, gene duplication or deep coalescence or noise derived from systematic or stochastic errors. In order to test the performance of the AHE datasets in a species complex of the genus *Lepanthes*, we conducted gene/species tree and network inferences together with phylogenetic informativeness analyses with concatenated and coalescent-based methods. We obtained a fully resolved phylogeny but also found high discordance in the topology of the individual gene trees and paraphyly in the grouping of alleles of one species, *Lepanthes horrida*. This might indicate that ancient hybridization, polyploidy and/or incomplete lineage sorting may have contributed to speciation in *Lepanthes*. These analyses also revealed two undescribed species that were not previously disclosed based on inferences from ITS and matK datasets. Our study shows that only with a large number of phylogenetic markers it possible to disentangle cryptic species and morphological traits evolving in parallel or convergently.

Evolution and development of *Phalaenopsis* flowers

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The size and shape of different orchid floral organs, such as the callus on the lip and stelidia along the column, serving as a holdfast for insects, evolved into a perfect fit with the bodies of specific pollinators. This morphological diversity is an attractive subject for studying the evolutionary basis of floral organ development, especially in the orchid genus *Phalaenopsis*. The objective of this study is to discover more about the evolution and development of the callus on the lip, and stelidia along the column for different species of the orchid genus *Phalaenopsis* that have large versus small calli and long versus short stelidia. Flowers of species such as *P. amabilis*, *P. equestris* and *P. pulcherrima* are currently being studied using Scanning Electronic Microscopy and 3D CT scanning. To investigate how their flowers fit with the bodies of their respective pollinators, scans of museum specimens of *Xylocopa* and *Amegilla* bees will be made and analyzed as well. Gene expression will be studied by transcriptome analysis and RT PCR of floral RNA to discover more about the evolution and development of specific floral organs enabling very precise placement of pollinia on specific body parts of pollinators.

What's the buzz about *Ophrys fusca* and *O. dyris*? Tittle-tattle between two bee orchids in central Portugal

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Ophrys is amongst the best known orchid genera, and is an established system for the study of pollinator-mediated floral evolution. Two species, *Ophrys fusca*. and *Ophrys dyris* (= *O. omegaifera* subsp. *dyris*) belonging to *Ophrys* section *Pseudophrys* are the focus of this study. Cases of introgression have been reported between these species, which have similar morphological characters and can be easily misidentified in the field. In order to better characterize the populations of these two taxa and its dynamics in central-Portugal, we integrated cytological and morphological and genetic diversity data between *O. fusca* and *O. dyris*, here focusing on the results regarding genome size, cytotype diversity and gene flow. Flow cytometry methods were used to assess genome size, and subsequently determine the ploidy level of 67 specimens, including the species and putative hybrids. Cytotypes were also confirmed based on chromosome counts from the roots of specimens of each species. Constancy of nuclear DNA content (1C = 11.19 pg) and ploidy level ($2n = 4x = 72, 74$) was documented among all the individuals analysed. Bayesian cluster analysis of 13 microsatellite loci and 167 individuals confirmed introgression and hybridization. Nevertheless, in this area of central Portugal, species seem to remain genetically circumscribed, as the number of genetic groups identified is two. Current results support the view of the Iberian Peninsula as a hotspot of polyploidisation in section *Pseudophrys*.

Biased frequency of short inverted-repeats in orchid chloroplast genomes

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Abstract – Genomes are structured by the sequences of nucleotides and their expression depend on various factors including chromatin condensation and methylation, repeated sequences, promotor structure and organization. Chloroplast and mitochondria genomes inherited from bacteria with their specific organization in circle shape and of small size making their analyze easier than that of nuclear genomes. Since the first complete sequence of chloroplast genome obtained for *Nicotiana tabacum*, many other chloroplasts genomes have been completely sequenced including in orchids. These genomes have been compared according their gene content and sequence but not really according to the distribution of short inverses repeats. In this study, we analyzed completely sequenced chloroplast genomes of 85 orchid species by counting the number of 2-, 4- and 6-bp short inverted repeats called palindromes. Their frequency is significantly lower than expected according to random association of nucleotides, this is particularly the situation for TA, CG, GC, ACGT, CGCG and TTAA while CCGG and especially GATC are significantly more frequent than expected. Species of Genus *Cypripedium* and *Epipogium* exhibited specific deviation from expectations, may be in *Epipogium* because of its small chloroplast genome size. Deviation of palindrome frequency within orchids fit only partly phylogeny. Palindrome sequences can be involved in interaction between DNA and proteins and consequently their frequency deviation can be related with gene expression, important cell process including relationships with foreign organisms.

INTRODUCTION

Chloroplast genomes are available for many plant species, including orchids. Mutations are supposed to be randomly distributed within genomes and shared point mutations are used to determine relationships among species and build phylogenies. Short inverted repeats consists of few DNA bases, like AACGTT able to form hairpins, whose DNA structure can be recognized and involved in expression control of genes. These sequences can be thus subjected to selection. The purpose of the present study is to determine if short inverted-repeats are randomly distributed in orchid chloroplast genomes.

MATERIALS AND METHODS

Analysis of 85 complete chloroplast sequences available in orchids:

- Counts for short-inverted repeats of 2, 4 and six DNA bases using seqinr package for R;
- Detection of biased distribution according to AT/GC content;
- Multivariate analyses of observed /expected frequency using FactoMineR package for R.

RESULTS

- Inverted-repeats: in average 9.7% less frequent than expected.
- Deviation in inverted-repeats distribution varies according to the species and sequence (Table 1).
- Most species have similar patterns excepted mainly *Epipogium* and *Rhizanthella* (Figure 1) which have a very reduced chloroplast genome size.
- Species cluster only partly according to phylogeny (Figure 2).

Table 1. Counts of some inverted-repeats in chloroplat genomes.

Taxon	2-bases	4-bases	6-bases	AT	TA	GC	ATAT	GATC	GCGC	GGCC	TATA	AACGTT	AGATCT	ATTAAT	TTCGAA
Anoectochilus emeiensis	36 177	9 395	2 542	15 407	12 050	4 406	1 469	792	121	237	1 143	171	67	88	82
Anoectochilus roxburghii	36 820	9 247	2 435	15 646	11 943	4 605	1 409	880	132	236	1 078	13	83	80	89
Aphyllorchis montana	22 562	5 736	1 589	9 668	7 395	2 700	928	515	86	141	725	93	53	51	54
Apostasia odorata	39 191	10 400	2 932	17 050	13 484	4 303	1 887	795	107	196	1 551	15	72	118	107
Apostasia wallichii	37 981	9 925	2 785	16 497	12 917	4 275	1 739	784	98	196	1 373	14	70	120	104
Bletilla ochracea	37 840	9 705	2 682	16 181	12 434	4 590	1 570	874	133	233	1 250	13	89	98	94
Bletilla striata	38 228	9 866	2 869	16 360	12 646	4 555	1 682	873	125	239	1 382	14	86	91	96
Calanthe triplicata	38 641	9 879	2 688	16 621	12 989	4 514	1 667	850	130	225	1 346	13	77	99	83
Cattleya crispata	35 350	8 884	2 398	15 187	11 621	4 253	1 451	831	127	217	1 110	12	76	82	81
Cephalanthera humilis	37 292	9 488	2 512	16 012	12 213	4 535	1 503	891	129	238	1 197	12	81	79	70
Corallorhiza macrantha	36 607	9 317	2 510	15 635	12 047	4 428	1 567	848	128	215	1 189	15	80	84	74
Corallorhiza trifida	36 149	9 187	2 475	15 436	11 917	4 388	1 548	849	123	219	1 186	13	85	90	80
Cymbidium ensifolium	35 914	9 236	2 564	15 457	11 848	4 277	1 536	828	120	212	1 207	14	77	103	79
Cypripedium formosanum	48 622	14 574	4 590	21 507	17 788	4 592	3 329	896	136	232	3 016	15	73	241	102
Cypripedium macranthos	37 807	9 691	2 572	15 856	12 283	4 655	1 451	886	131	249	1 109	19	82	107	100
Dendrobium parciflorum	35 463	8 937	2 414	15 080	11 517	4 360	1 426	842	126	227	1 088	13	85	83	88
Elleanthus sodiroi	38 556	9 801	2 653	16 540	12 767	4 546	1 595	897	126	225	1 225	14	86	99	100
Epipactis veratrifolia	37 999	9 649	2 607	16 275	12 451	4 618	1 540	870	130	243	1 176	12	69	89	85
Epipogium aphyllum	9 558	2 899	908	3 831	3 687	1 021	650	82	41	30	565	3	2	69	11
Epipogium roseum	5 862	1 836	631	2 345	2 419	575	406	37	17	18	414	3	4	32	7
Erycina pusilla	34 456	8 716	2 323	14 724	11 550	4 042	1 313	790	121	193	1 062	14	84	88	84
Gastrochilus fuscopunctatus	34 996	9 003	2 420	15 067	11 665	4 123	1 450	811	118	209	1 157	10	83	100	78
Gastrochilus japonicus	35 209	9 094	2 435	15 163	11 703	4 162	1 462	828	115	208	1 153	9	83	86	80
Goodyera velutina	36 108	9 313	2 523	15 428	11 951	4 423	1 489	797	122	237	1 133	14	65	93	76
Habenaria pantlingiana	37 668	9 806	2 704	16 104	12 660	4 485	1 595	821	125	230	1 346	12	75	99	88
Liparis loeselii	37 289	9 385	2 542	15 890	12 476	4 458	1 470	837	134	217	1 188	14	74	94	71
Listera fugongensis	36 893	9 353	2 534	15 718	12 023	4 602	1 431	881	141	242	1 102	13	82	86	78
Ludisia discolor	35 937	9 295	2 525	15 301	11 910	4 405	1 443	798	118	236	1 105	13	72	93	87
Masdevallia coccinea	38 086	9 579	2 560	16 314	12 785	4 489	1 540	875	130	222	1 171	13	85	100	81
Neottia acuminata	19 680	5 028	1 383	8 225	6 713	2 427	768	432	76	108	610	6	38	53	27
Neottia camtschatea	25 214	6 401	1 739	10 730	8 342	3 111	1 002	628	102	150	801	8	57	69	45
Neottia nidus-avis	23 861	6 485	1 850	10 393	8 798	2 447	1 240	458	53	111	1 119	5	40	81	26
Neottia ovata	36 885	9 310	2 495	15 717	11 987	4 611	1 392	877	134	242	1 072	12	76	87	89
Neuwiedia singapureana	40 434	11 013	3 285	17 526	13 988	4 447	2 024	820	113	214	1 840	13	68	136	96
Oberonia japonica	34 072	8 433	2 230	14 484	11 291	4 154	1 306	798	131	198	974	14	74	92	79
Oncidium sphacelatum	35 380	9 044	2 419	15 162	11 672	4 218	1 448	822	121	204	1 147	13	81	97	92
Paphiopedilum niveum	40 428	11 095	3 276	17 573	14 045	4 386	2 106	857	119	224	1 837	13	68	147	81
Pelatantheria scolopendrifolia	35 433	9 293	2 563	15 347	11 881	4 104	1 577	826	113	216	1 250	11	78	112	80
Phalaenopsis aphrodite	35 654	9 262	2 497	15 368	11 920	4 191	1 495	826	114	222	1 178	10	82	95	78
Phragmipedium longifolium	38 405	10 454	2 955	16 518	13 267	4 254	1 912	817	127	208	1 623	11	65	115	88
Rhizanthella gardneri	16 218	4 406	1 317	7 005	5 907	1 648	834	317	70	79	764	11	35	52	33
Sobralia callosa	38 751	9 871	2 716	16 638	12 867	4 548	1 617	901	129	226	1 284	13	90	90	103
Thrixspermum japonicum	36 544	9 682	2 702	15 852	12 465	4 116	1 736	810	114	208	1 383	10	82	116	77
Vanilla aphylla	38 553	10 466	3 042	16 835	13 484	3 955	2 108	741	104	206	1 832	13	79	132	112
Vanilla planifolia	36 941	9 964	2 866	16 274	12 517	3 911	1 912	755	109	194	1 543	15	83	111	125

α = 0.0001 α = 0.001 α = 0.01
Observed counts > expected counts

α = 0.0001 α = 0.001 α = 0.01
Observed counts < expected counts

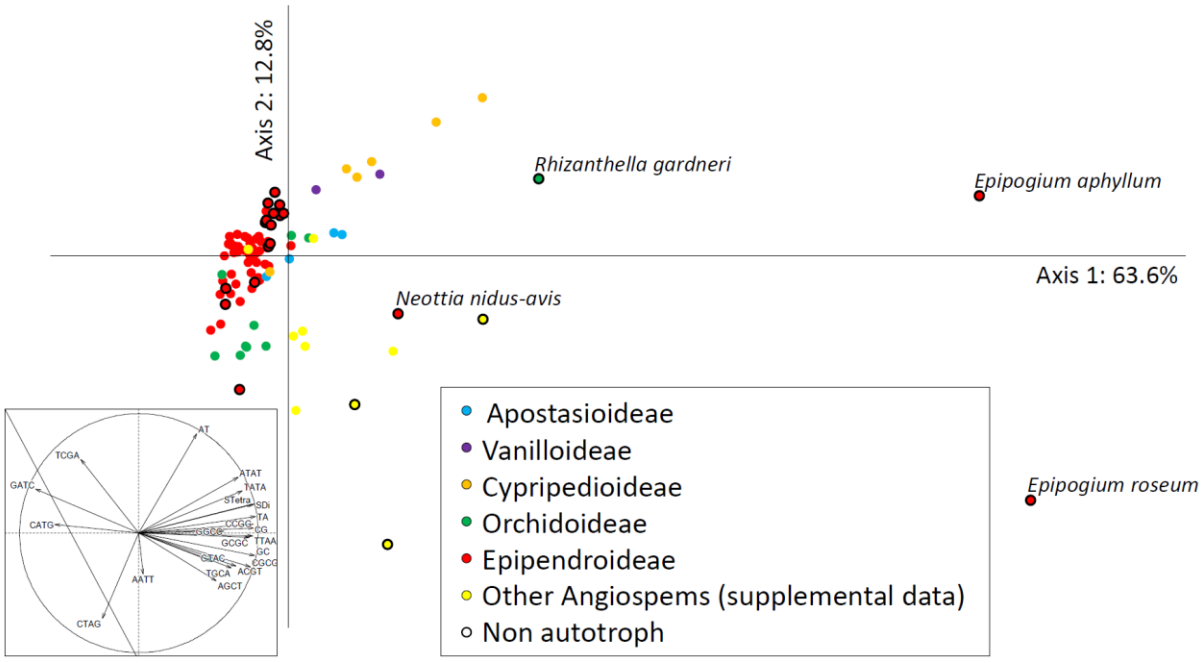

Figure 1. Principal component analysis applied on inverted-repeats of chloroplast genomes.

CONCLUSION

Some inverted-repeat sequences are significantly more frequent than expected while some other ones are less frequent in chloroplast genome, independently from phylogeny. Their frequency appeared thus under selection pressure and possibly related to adaptation. *Epipogium* and *Rhizanthella*, underground orchids, have a peculiar pattern of inverted-repeats in complement to their reduced chloroplast genome size. Deviation from expected distribution of inverted-repeats shows a signature for plant habitus according to their autotrophy pattern at adult stage. It may be related to their ability to contract relationships with other organisms (may be not only mycorrhizae).

Such selective effetcs on short DNA sequences could introduce deviation in phylogeny building (reduced probability to get TA, CG and CG succession in DNA bases).

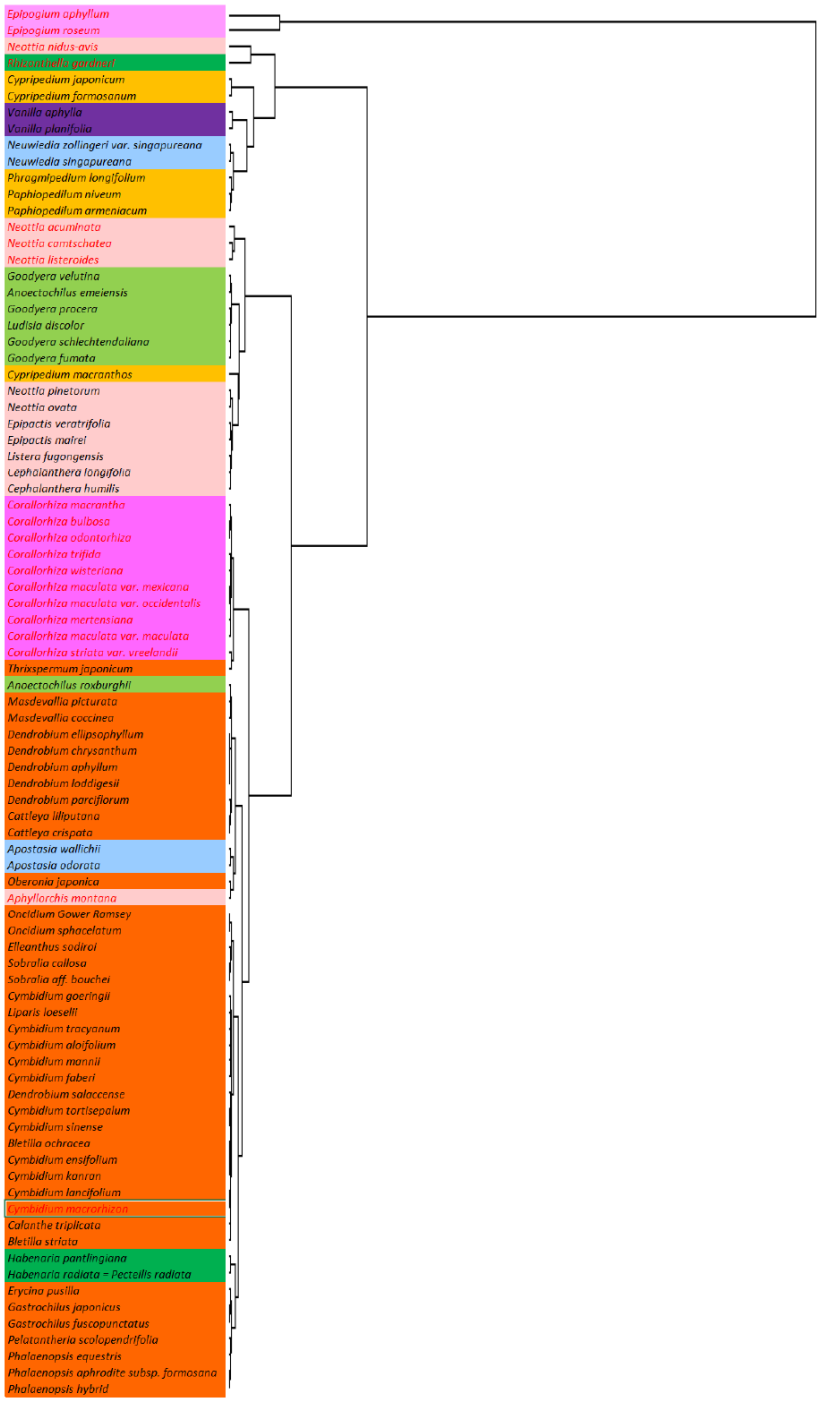

Figure 2. Hierarchical cluster analysis applied on inverted-repeats of chloroplast genomes.

RNAseq as a source of genetic polymorphisms in *Epipactis* for molecular marker development

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Abstract – Most orchids have large genomes and consequently the development of molecular markers to analyze their genetic diversity is rather difficult. Isozymes have firstly been used, but now due to the evolution of techniques and the hazardous chemicals required, this type of genetic markers is no longer considered. Microsatellite markers are codominant markers and suitable for genetic analyses but their development is difficult and costly especially for large genome species. Consequently, genetic analyses in orchids have been restricted to few species and with a limited number of markers. New genome sequencing methods became very efficient and of reduced cost. In this context, in order to limit the sequencing effort, in *Epipactis*, we have investigated sequencing of coding and expressed sequences by RNAseq. cDNA fragments have been standardized to a length of 450 pb and then sequenced. In order to increase the level of polymorphisms, three plants, two from *Epipactis helleborine* and one from *E. placentina* were investigated. Both plants of *E. helleborine* were taken in separated stands, one at low elevation (200 m) and one at higher elevation (2300 m). We analyzed bulked flowers within a large developmental gradient from buds to pollinated flower in order to increase the number of expressed genes. Sequences of approximatively 200 DNA bases at each ends were obtained by 454 sequencing. About 6 billion of sequences per plant have been assembled into 100 000 sequences using trinity software. Obtained sequences are almost similar to already known sequences obtained in Monocots. Assembled gene sequences from the three different plants have been aligned in order to find sequence polymorphisms. Polymorphic sequences have been detected and are suitable to design primer pairs in order to reveal genetic variation within and among species in *Epipactis*. The next steps will consist of these primer pairs test and genetic analysis within and among populations.

INTRODUCTION

Genetic studies are required to provide suitable information for orchid conservation but the development of molecular markers is rather difficult in orchids due to their large genome size. Recent evolution of molecular biology techniques with new generation sequencing provides tools for analyzing polymorphism. Among them, RNA-Seq allows to focus analysis on expressed sequences and to reduce amount of sequencing effort. Gene sequences can be reconstructed by assembling short sequences. Gene variation can be obtained by sequence comparison of both alleles of heterozygous genes. In order to increase the level of detected polymorphisms, three different plants belonging to two different species and from ecologically different stands are investigated. Molecular markers will be then developed and applied for genetic studies.

MATERIALS AND METHODS

- Plant materials: two plants of *Epipactis helleborine* (210 m asl, poplar plantation; 1300 m asl, meadow) and one plant of *E. placentina*; flowers (flower buds to wilted flowers) were collected and bulked separately for each plant (immediate storage in liquid nitrogen).

- Messenger RNAs were extracted and fragmented (average of 400 bases) prior to reverse transcription, amplification and Illumina sequencing (150 bases from each end; Figure 1).

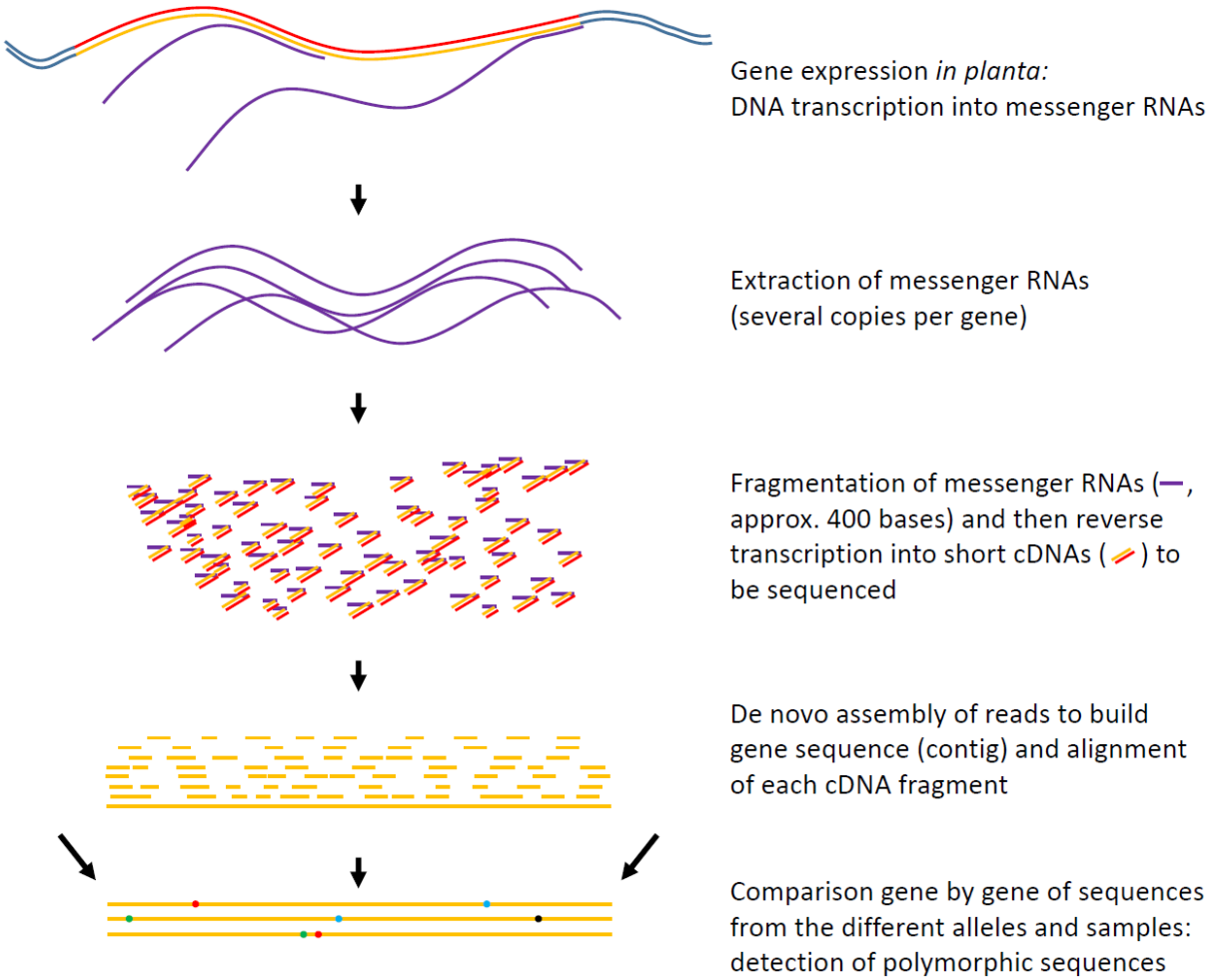

Figure 1. Principle of the present study.

- Sequences were assembled with Trinity (Galaxy platform). Reads of each plant were aligned with BWA on complete assembly. Sequences and polymorphisms were analyzed with Galaxy platform tools (Mpileup, varscan…).

RESULTS

- More than 4 000 000 fragments were sequenced for each plant (Table 1); their cumulated length represented more than 1 450 000 000 pb.

- The complete assembly consists of about 200 000 contigs, of an average size of 605 bp and median size of 345 pb stretching on 120 000 000 aligned bases. Sequencing depth reached thus about 12x.

- Sequence similarity analyses by blast*n* and Diamond reveal long assembled chloroplast genome sequences (up to 10 054 bp) and the identification of genes for more than 11 000 proteins, some hits being related to retrotransposons

- Polymorphisms was detected at the level of individual plant (related to heterozygosity) and at the complete sample (Table1). A large amount of SNPs was detected, more than 1.6 SNP/kbp. Indels were less abundant.

- *E. placentina* sample was not particularly differentiated.

- Position of SNPs and indels are localized along complete assembled sequences (Figure 2).

CONCLUSION

RNA-Seq analysis reveals a large amount of polymorphisms suitable for genetic studies. It provides also information on expressed genes.

Polymorphisms observed on coding sequences could be more related to plant phenotype and more reliable to morphological variation among plants and species.

Next and important steps would be design of primer pairs and DNA amplification in order to test their ability to reveal gene diversity. Selected primers will be then applied in *Epipactis* genetic studies.

Table 1. Main features of RNA-Seq analysis.

	E. helleborine 1	*E. helleborine* 2	*E. placentina*	Complete dataset
Reads	5 651 111	4 641 189	4 373 442	14 665 742
Number of contigs	102 452	100 463	96 786	199 373
Coding DNA sequences	68 276	63 968	61 778	118 200
Single Nucleotide Polymorphisms	17 157	16 311	9 586	71 131
Polymorphic contigs (counts)	5 478	4 812	3 240	17 739
Polymorphic contigs (%)	5.3	3.3	4.8	8.9
Contigs with indels	900	866	633	3 283

Parameter *Minimum read depth* set to 25

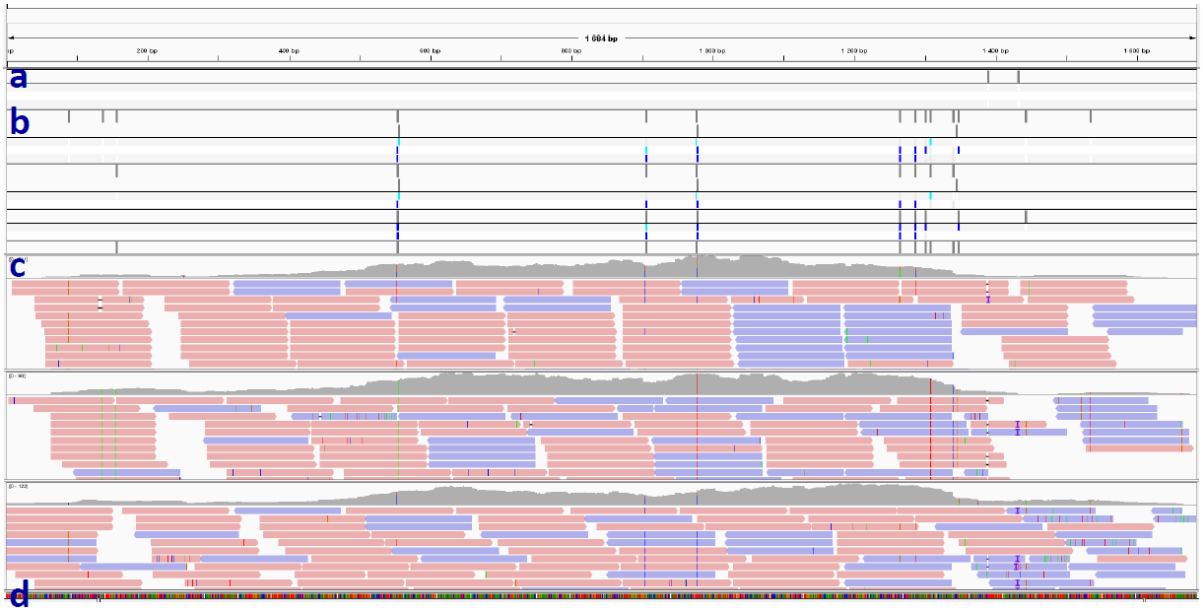

Figure 2. Position of indels (a), SNPs (b) and of reads for the three plants (c) along assembled sequence (d) of aminoacyl tRNA synthase complex-interacting multifunctional protein 1 (1684 pb).

Orchid biotechnology and breeding

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Abstract – Containing more than 25,000 species, the Orchidaceae family, is one of the largest angiosperm families. The genus *Phalaenopsis*, a beautiful and popular orchid, comprises approximately 66 species. The nuclear DNA contents and karyotypes analysis from 18 *Phalaenospsis* species have been estimated by using flow cytometry and cytogenetic analysis, respectively. OrchidBase 3.0 collects the transcriptomics data of ten species distributed in the five subfamilies as well as the whole genome sequence of the tropical epiphytic crassulacean acid metabolism (CAM) orchid, *P. equestris*, a frequently used parent species for orchid breeding. *P. bellina* is a scented orchid emitting large amount of monoterpenes. GERANYL DIPHOSPHATE SYNTHASE (PbGDPS) is the key enzyme for monoterpene biosynthesis. A dual repeat in the upstream promoter fragments of *GDPS* is essential for its transcriptional activation in *Phalaenopsis* orchids. The full dual repeat was present only in the scented *Phalaenopsis* orchids, and its integrity showed strong association with the transactivation by a basic leucine zipper (bZIP) TF. As this dual repeat was close related to the monoterpene biosynthesis in *Phalaenopsis* orchids, it could be developed as a promising molecular marker for early detection of monoterpene phenotype in the offspring and thus facilitate scented orchid breeding. In addition, we have performed genotyping-by-sequence for *Phalaenopsis* genotyping from the cross between *P. aphrodite* ssp. *formosana* and *P. equestris* and their 118 F1 progenies, and set up the bioinformatics system for the marker-assisted selection on molecular breeding and gene identification.

Keywords: breeding, bZIP, dual repeat, orchid biotechnology, PbGDPS, *Phalaenopsis*

With an estimated more than 25 000 species, orchids are the most species-rich of all angiosperm families. They show a wide diversity of epiphytic and terrestrial growth forms and have successfully colonized almost every habitat on earth. The most recent common ancestor of extant orchids lived in the late Cretaceous (76-84 Mya) as dated by a fossil orchid and its pollinator (Ramirez *et al*., 2007). The radiation of the orchid family has probably took place in a comparatively short period as compared with that of most flowering plant families, which suggests that their speciation rates are presumed to be exceptionally high (Gill, 1989).

Associated with the enormous number of Orchidaceae species is extraordinary floral diversification. Orchids are renowned for an abundance of kinds, with a seemingly unending array of strange and often fantastic variations, and represent a highly advanced and terminal line of floral evolution in the monocotyledons. This spectacular diversification has been linked to the specific interaction between the orchid flower and pollinator (Cozzolino and Widmer, 2005), sequential and rapid interplay between drift and natural selection (Tremblay *et al*., 2005), the role of obligate orchid-mycorrhizal interactions (Otero and Flanagan, 2006), and Crassulacean acid metabolism and epiphytism (Silvera *et al*., 2009). In addition to their prosperity of ecological manipulations, orchids have several unique reproductive strategies that contribute to their success. These include mature pollen grains packaged as pollinia, pollination-regulated ovary/ovule development, synchronized timing of micro- and mega-gametogenesis for effective fertilization, and the release of thousands or millions of

immature embryos (seeds without endosperm) in mature pods (Yu and Goh, 2001).

Because of the thriving and prosperous orchid breeding and industry, plant scientists in Taiwan are well placed to study orchid biology and develop orchid biotechnology to apply to the orchid industry.

A better understanding of the karyotypes and DNA contents of orchid will aid in the development of new cultivars of orchids. All *Phalaenopsis* species have the same chromosome number ($2n = 2x = 38$), but their genomes vary considerably in size. Analysis of karyotypes of 9 *Phalaenopsis* species and *Doritis pulcherrima* by Feulgen- and DAPI-stained somatic metaphase chromosomes from root tips revealed that *P. aphrodite*, *P. stuartiana*, *P. equestris*, *P. cornu-cervi*, and *P. lueddemanniana* are with small and uniform chromosomes (1-2.5 μm), and all are metacentric or submetacentric. *P. venosa*, *P. amboinensis*, and *P. violacea* have bimodal karyotypes, with large and small chromosomes, and most are subtelocentric or acrocentric (Kao *et al*., 2001). Flow cytometry has proven to be an efficient and reliable method for analyzing plant genomes. The nuclear DNA contents from 18 *Phalaenospsis* species and *P. pulcherrima* are estimated by flow cytometry; 2C values ranged from 2.74 pg for *P. sanderiana* to 16.61 pg for *P. parishii* (Lin *et al*., 2001).

The OrchidBase collects the transcriptome sequences from *Phalaenopsis* cDNA libraries and assembled into 84 617 non-redundant transcribed sequences (including 8 501 contigs and 76 116 singletons) (Fu *et al*., 2011). The OrchidBase contains the transcriptome sequences derived from 11 *Phalaenopsis* orchid cDNA libraries, which are constructed from different species, including *P. aphrodite* subsp. *formosana*, *P. equestris* and *P. bellina*, and from different tissues, including developing seed, protocorm, vegetative tissue, leaf, cold-treated plantlet, pathogen-treated plantlet, inflorescence, and flower buds (Fu *et al*., 2011). The transcriptomics data collected in OrchidBase 2.0 are obtained from 10 orchid species within 5 subfamilies through both deep sequencing with ABI 3730 and NGS Roche 454 and Illumina/Solexa. Recently, the whole genome sequences are available for *P. equestris*, *Dendrobium catenatum*, and *Apostacea shengenica*, and included in the OrchidBase 3.0. The OrchidBase is freely available at http://orchidbase.itps.ncku.edu.tw and provides researchers with a high-quality genetic resource for data mining and efficient experimental studies of orchid biology and biotechnology.

The global flower industry thrives on novelty. Domestication of wild species in conjunction with traditional breeding has long been the principle path for generation of novel flowers in the industry. For orchid, traits such as flower color, shape and fragrance are primary novel markers because they are key determinants of consumer choice. However, many modern floricultural varieties have lost their scent with traditional breeding programs. Breeders of orchids in cut-flower and ornamental markets have focused on producing plants with improved vase life, shipping characteristics and visual aesthetic values (*i.e*., color and shape).

The growing cycles of *Phalaenopsis* orchids are 2-3 years. Using traditional hybridization to transmit useful traits into commercial varieties is a long process that will take years to achieve (Arditti, 1992). In addition, intraspecific and/or interspecific incompatibility limits the work of variety improvement. All 5 subgenuses of *Phalaenopsis* have the same chromosome number (2n=2x=38) that can be divided into small, medium and large chromosome groups, according to chromosome sizes and nuclear DNA contents (Kao *et al*., 2001, Lin *et al*., 2001). Most commercial cultivars are derived from species with small chromosomes, such as *P. amabilis*, *P. aphrodite*, *P. stuartiana*, *P. schilleriana*, and *P. equestris*. The species with strong scents have large chromosomes including *P. amboinnensis*, *P. bellina*, *P. venosa* and *P. violacea*. Successful crosses between species with small and with large chromosomes are difficult because of inter-specific incompatibility.

P. bellina, classified in the subgenus *Polychilos*, is native to Malaysia, and numerous commercial varieties have been bred because of the orchid's pleasant fragrance. In addition, the species has some native tetraploid species to breed scented commercial *Phalaenopsis* orchids and therefore is an important parent for breeding scented cultivars. Floral scent is a composite characteristic determined by a complex mixture of low molecular mass volatiles molecules and dominated by monoterpenoid,

sesquiterpenoid, phenylpropanoid, benzenoid compounds and fatty acid derivatives. The floral scents in *P. bellina* are rich in monoterpenes, geraniol and linalool and their derivatives (Hsiao *et al*., 2006). They include geraniol, nerol, 2,6-dimethyl-octa-3,7-diene-2,6-diol, 2,6-dimethyl-octa-1,7-diene-3,6-diol, 3,7-dimethyl-2,6-octadienal, geranic acid and 2,6-dimethyl-octa-2,6-diene-1,8-diol. In contrast, no monoterpenoid derivatives were emitted in scentless *P. equestris* flowers; fatty acid derivatives, phenylpropanoids, and benzenoids were the major volatiles. These compounds are barely detectable by the human nose.

Research into plant scents has been hampered mainly by the invisibility of this character, its dynamic nature, and complex mixtures of components that are present in very small quantities. Combining chemical analysis, genomics and bioinformatics, we have uncovered the scent biosynthesis pathway and the relevant genes in *P. bellina* flower. These include a monoterpene biosynthesis pathway of 15 steps in the *P. bellina* flower leading from glyceraldehyde-3-phosphate to monoterpenoids of geraniol, linalool and their derivatives (Figure 1, Hsiao *et al*., 2006).

Terpenoids belong to a large family of plant secondary metabolites, and their corresponding alcohols possess useful properties such as fragrance, flavour, insecticidal properties and characteristics that make them useful as pharmaceutical agents. All monoterpenes are derived from the same substrate, geranyl diphosphate (GDP, C_{10}), which is catalyzed by GDPS, a member of the short-chain trans-prenyltransferase family, via the condensation of dimethylallyl diphosphate with isopentenyl diphosphate. GDPS (Tholl *et al*., 2004) is differentially expressed in the scented species.

The full-length cDNA of *P. bellina* GDPS (PbGDPS) was isolated from a *P. bellina* floral cDNA library (Hsiao *et al*., 2006) and sequenced. *PbGDPS* was predominantly expressed in the scented species *P. bellina* and its scented offspring D. Kenneth Schubert 'Five'. In addition, its expression level was associated with the amount of scent emitted in the scented species, suggesting that *PbGDPS* play a key role in the regulation of scent production in *P. bellina* flowers (Hsiao *et al*., 2008).

Comparing the promoter sequence of from scent species *P. bellina* and scentless species *P. aphrodite*, we found that there is a dual repeat consisted of two 75 bp units present in the scent *Phalaenopsis* species. We hypothesized that the dual repeat is associated with the monoterpene production. To confirm this, another 10 *Phalaenopsis* orchids frequently used as breeding parents (Figure 2) were assessed for the correlation analysis between the dual repeat and the monoterpene production. The presence of the GDPS gene and its promoter sequence in the 12 *Phalaenopsis* orchids were then analyzed. Intriguingly, the GDPS gene was all present in these orchids regardless of their scent or scentless phenotype. It is plausible that the defects are resided in the promoter region. We then amplified the dual repeat and found its fragment length polymorphism among the 12 *Phalaenopsis* orchids. The four scented orchids with monoterpene production contain the complete dual repeat (Figure 3, the black arrowheads). In contrast, the length of the amplified fragments of the other orchids were reduced to various extents due to deletion in the dual repeat region (Chuang *et al*., 2018).

Furthermore, the dual repeat was used to screen the transcription factor library and identified the PbbZIP4. We found that PbbZIP4 was able to distinguish whether the promoter containing the dual repeat. There are two promoter fragments of *PaGDPS* in *P. aphrodite*, namely PaGDPSpA and PaGDPSpB. In the presence of PbbZIP4, it enhanced the promoter activities of both PbGDPSp and PaGDPSpA which have complete dual repats, but showed no effects on PaGDPSpB which does not have the complete dual repeat. Collectively, these results indicated that the upstream activator, bZIP4, as well as the dual repeat of *GDPS* promoter are crucial for monoterpene production in *Phalaenopsis* orchids (Chuang *et al*., 2018).

We performed genotyping-by-sequence (GBS) approach by using restriction-site associated DNA sequencing (RAD-Seq) to construct a high-density genetic map for for *P. aphrodite* ssp. *formosana* x *P. equestris* and their 118 F1 progenies. The traditional SSR marker-assisted genetic map and the reference whole genome sequence of *P. equestris* have been improved to assemble into 19 linkage groups to assist SNP mapping. Totally, 2 819 SNPs were mapped to the 19 linkage groups derived from the *Phalaenopsis* genome with the averaged 311 kp sequence containing a

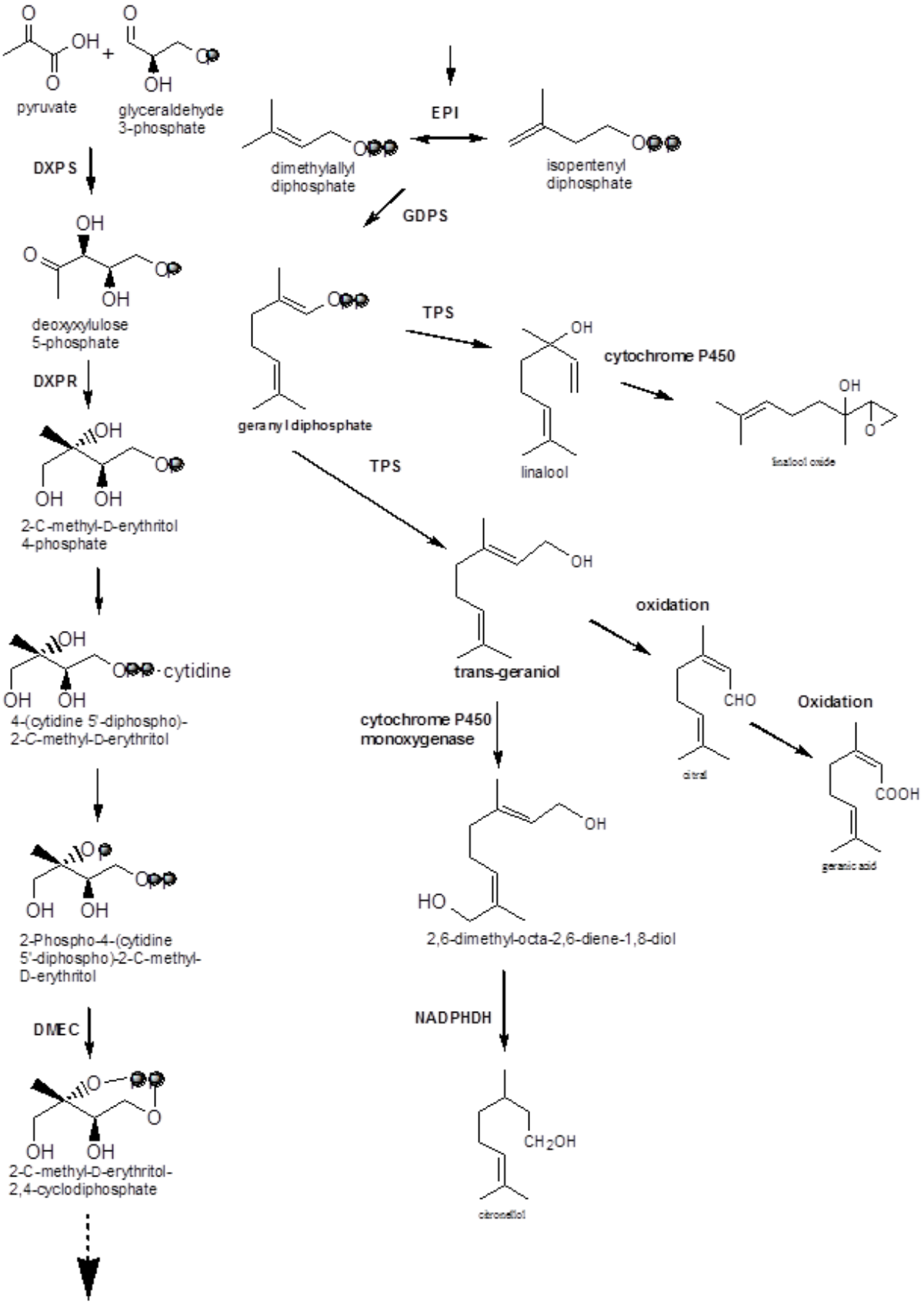

Figure 1. Putative metabolic pathway from pyruvate and glyceraldehyde-3-phosphate to scent synthesis and related enzymes in *P. bellina*. DXPS: deoxyxylulose-5-phosphate synthase; DXPR: deoxyxylulose-5-phosphate reductoisomerase; DMEC: 4-diphosphocytidyl-2-C-methyl-D-erythritol 2-phosphate cyclase; EPI: epimerase; GDPS: geranyl diphosphate synthase; NADPHDH: NADPH dehydrogenase (Adopted from Hsiao *et al*., 2006).

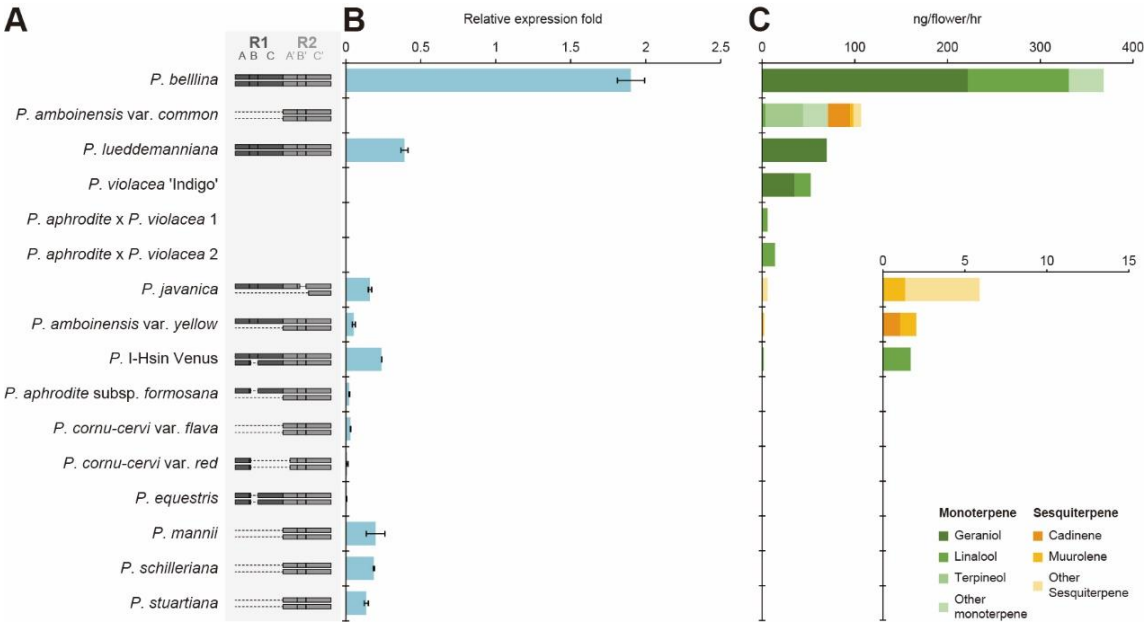

Figure 2. The 12 *Phalaenopsis* orchids used in this study. The order of the figures was followed the presentation in Figure 2. **(A)** *P.* Meidarland Bellina Age 'LM128', **(B)** *P. bellina*, **(C)** *P. lueddemaniana*, **(D)** *P.* I-Hsin Venus, **(E)** *P. javanica*, **(F)** *P. amboinensis* var. *yellow*, **(G)** *P. mannii*, **(H)** *P. schilleriana*, **(I)** *P. aphrodite* subsp. *formosana*, **(J)** *P. cornu-cervi* var. *red*, **(K)** *P. equestris* 'RO-5', and **(L)** *P. equestris* 'WY-7'. Scale bar = 1 cm.

SNP marker. GWAS analysis was performed for these 2 819 SNPs with various agricultural traits in the F1 population of *P. aphrodite* ssp. *formosana* x *P. equestris*, including flower size, sepal size, and petal color, and the functions of these genes near these SNPs were further confirmed. Therefore, we have developed a draft of genetic map that can be helpful for the genetic study in *Phalaenopsis* and benefit for orchid breeding (Chiou *et al*., unpublished data).

Funding

This work was supported by Ministry of Science and Technology, Taiwan. [Grant no. 105-2313-B-006 -002 -MY3 and 106-2321-B-006 -009 to H.H.C].

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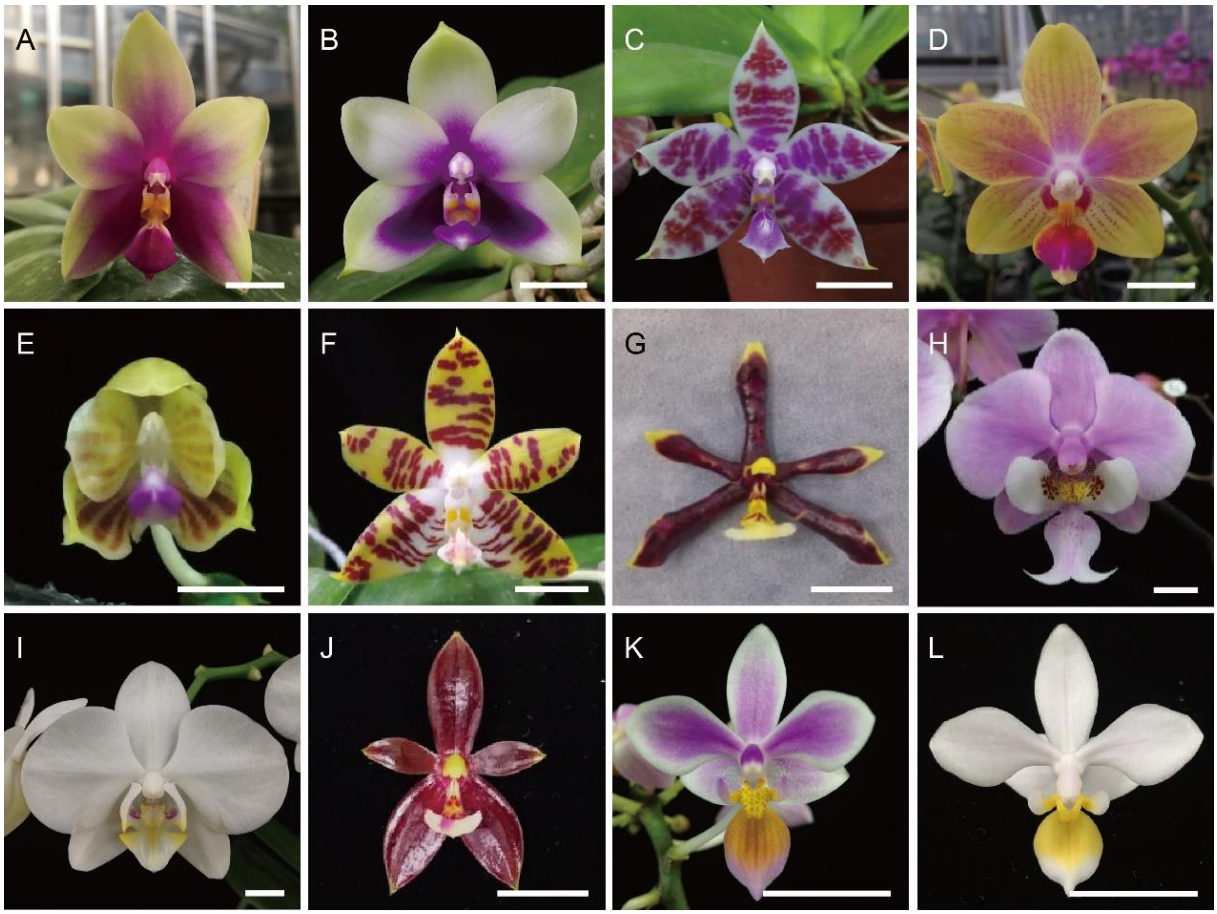

Figure 3. The analysis of *GDPS-SSU1* gene and floral volatiles in native *Phalaenopsis* orchids and hybrids. The R1R2 region structure of *GDPS-SSU1* promoter **(A)**, the expression levels of GDPS-SSU1 **(B)**, and floral volatile analysis **(C)** of native *Phalaenopsis* orchids and hybrids. Data is from single plant measurements.

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Achievements of *Phalaenopsis* orchid breeding in Taiwan

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Phalaenopsis is the most popular cultivated orchids in the world because of its infinite inflorescence with many beautiful and long-lasting flowers. There are 92 wild species and 34,112 hybrids of *Phalaenopsis* registered in the Royal Horticultural Society (RHS) in 2017, although only about 18 species are most used for breeding. Taiwan orchid breeders create and produce most commercial varieties for the market. The major species contributing to the large flowers are *P. amabilis* and *P. aphrodite*, accounting for more than 90% bloodline. *P. aphrodite* and *P. equestris* are only found in Taiwan which are very important species to breed new hybrids with multi-flowers. Up to 2017, *P. aphrodite* has produced 58 G1-generation hybrids (First generation progenies) and a total of 31,460 hybrids in 12 generations. *Phalaenopsis* Timothy Christopher (*Phalaenopsis* Cassandra x *P. aphrodite*) has an average of 35.0 flowers per spike with a 5.5-cm flower size, and produced 197 next-generation hybrids and a total of further 615 hybrids. *Phalaenopsis* Doris, containing 12.50% germ of *P. aphrodite*, is a very important breeding line for large flowers and has produced a total of 30,266 hybrids. The most famous hybrid, *Phalaenopsis* Sogo Yukidian 'V3' which contains 15.30% of *P. aphrodite* and 53.03% of *Phalaenopsis* Doris germ, respectively, almost represents the large-and-white flower in *Phalaenopsis* market. *P. equestris* has short spikes and many small flowers. There are 550 G1-generation hybrids and 21,805 hybrids derived from *P. equestris* in 13 generations. *Phalaenopsis* Cassandra (*P. equestris* x *P. stuartiana*) is an important G1-generation hybrid produces 222 next-generation hybrids and a total of further 3,305 hybrids. Among the 13 generations, the G2 to G10 generations produced the higher number of hybrids and had over 1,000 hybrids for each generation, especially each of G6 to G8 generations containing more than 3,000 hybrids. Recently, the breeding of short spikes with many-flowered cultivars has been gaining popularity, because these cultivars are plastic for table decorations and cost-save for the growth space and transport. Therefore, the use of progenies from *P. equestris* and *P. aphrodite* for breeding parents would be continued and lasted for a long time. Furthermore, harlequin flowers and big-lipped flowers are currently important *Phalaenopsis* cultivars in the world. The harlequin flowers are always shown as clown-spot pattern, and result in very complicated color patterns. The occurrence of harlequin flowers came from the finding of somaclonal mutants of *Phalaenopsis* Golden Peoker 'Brother'. The beginning of the big-lipped flowers was from the occurrence of *Phalaenopsis* World Class 'Big Foot'. The progenies of its G1 hybrids, *Phalaenopsis* Yu Pin Easter Island and *Phalaenopsis* Yu Pin Fireworks are the major breeding parents for big-lipped hybrids. In addition, the sizes and numbers of chromosomes in plant cells affect the crossing efficiency and are the bottleneck for a breeding program. We will present a chromosome doubling technique to develop the strategy of polyploidy breeding. Therefore, the breeding for *Phalaenopsis* has been extending to very wide diversity.

Vanilla: a challenging genus with regards to the development of genomic resources

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The development of enabling genomic resources for *Vanilla* would dramatically advance international efforts to improve the genetic foundation of this important global commodity for vanilla-growing countries worldwide. Currently, global production of *Vanilla planifolia* rests on a precarious genetic foundation that lacks natural resistance to disease-causing pathogens (such as *Fusarium*) and environmental fluctuations. This low genetic diversity of cultivated vanilla leaves it highly vulnerable to disease, climatic change, and other environmental stresses, placing the entire vanilla industry potentially at risk.

However, *Vanilla* is a challenging genus with regards to development of genetic resources: not only do *Vanilla* species have large genomes (approx. Cx = 2.5 Gb), but they are also characterized, like some orchids from various sections, by strict partial endoreplication (SPE) cycles, unknown in any other plant family.

We will present how we took these major constraints into account in the definition of genome sequencing strategies for this genus

The transcriptomic and proteomic profile of symbiotic germination of *Dendrobium officinale* (Orchidaceae)

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Mycorrhizal fungi colonize orchid seeds and induce the germination. This so-called symbiotic germination is a critical developmental process in the lifecycle of all orchid species. However, little is known about the molecular changes taking place during seed germination, especially symbiotic germination. *Dendrobium officinale* is an endangered epiphytic orchid, which is widely used in traditional Chinese medicine in China. In previous studies, we have screened the fungi (*Tulasnella* sp.) promoting germination of *D. officinale* and examined the ultrastructural changes accompanying symbiotic germination. Here, our comparative transcriptomic and proteomic analysis between asymbiotic and symbiotic germination of *D. officinale* seed revealed fungal colonization of orchid seeds appears to induce higher and earlier expression of some key proteins involved in lipid and carbohydrate metabolism and thus improves the efficiency of utilization of stored substances present in the embryo. Phytohormone quantification revealed plant hormone accumulation in the protocorm of *D. officinale* infected fungi. Exogenous GA treatment or adding GA inhibitor can inhabit mycorrhizal formation and decrease seed germination rate in symbiotic germination assay. Therefore, we supposed that plant hormone involved the crosstalk signal pathway between hormone biosynthesis and common symbiotic signal pathway during seed symbiotic germination of *D. officinale*.

Cloning and characterization of a 9-cis-epoxycarotenoid dioxygenase genes in developing seeds of a tropical terrestrial orchid, *Phaius tankervilliae*

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Orchid seeds are characterized by their tiny size, with a globular stage embryo and without endosperm. Seed germination in vitro of some terrestrial orchids is difficult as the seed matured. Previous studies have shown that the endogenous ABA level of seeds remained high at maturity, suggesting that the low germination percentage may be caused by the accumulation of high level of ABA in mature seeds. In developing seeds of *Phaius tankervilliae,* we cloned a gene, with predicted protein sharing high sequence similarity with the 9-cis-epoxycarotenoid dioxygenase (NCED) that was up-regulated as the seed approached maturity. Southern hybridization confirmed that this NCED, named as PtNCED1, is a single copy gene in *P. tankervilliae*. As NCED catalyze the conversion of 9-cis xanthophylls to xanthoxin, an ABA precursor, it is speculated that the un-descending NCED transcripts in seeds may cause the accumulation of endogenous ABA. Transient expression of PtNCED1 gene in tobacco leaves resulted in the accumulation of ABA as compared to the control.

The challenges and successes achieved in the Chikanda orchid project

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In order to propagate the required orchids for the Chikanda Orchid project, adequate training is required for successful growth of plants both in the laboratory and in the nursery. A review of the practical issues and challenges involved in teaching *in vitro* methods and procedures developed for the conservation of the chikanda orchids in the laboratory. Various hands on methods taught in the nursery to ensure survival of collected mature plants and hardened off plants out of the laboratory. Without correct procedures followed, conservation efforts would be thwarted.

Project Location: Copperbelt University, Zambia
Funded by: Darwin Initiative

Disa barbata and other terrestrial orchids of the Fynbos biome - surprises and experiences

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Terrestrial orchids are a significant component of the fynbos biome. They count amongst the most beautiful, intriguing and desirable plants of the fynbos. They occupy a variety of habitat niches within the biome and culturally are greatly affected by fires, floods and human encroachment. Understanding their cultural needs helps clarify how these plants survive. The *Disa barbata* project has been the flagship project to understanding how these plants develop through the various stages from seed to mature flowering plant.

The hairy taxonomy of *Eria*

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Until recently, *Eria* was one of the big three orchid genera in Asia, with about 350 species (the two others being *Bulbophyllum* and *Dendrobium*). Molecular phylogenetic studies have since shown that *Eria* is polyphyletic. Its species are distributed over three main clades, interspersed with several other genera, such as *Appendicula*, *Ceratostylis*, *Mediocalcar*, *Porpax*, and *Pseuderia*. Evidently, the generic circumscription of *Eria* needed revising. Based on ITS, matK, trnL-F spacer, trnL intron and ycf1 sequences a phylogenetic hypothesis for tribe Podochileae with emphasis on subtribe Eriinae s.s. was proposed. The resulting tree showed that *Eria* could be divided into smaller genera along the lines of many of the previously recognized sections, with some exceptions. It turned out that some of the traditional sections of *Eria*, e.g. sect. *Hymeneria*, were themselves polyphyletic. Generic delimitation thus presented a dilemma: either to lump many morphologically distinctive genera into a single supergenus much larger than *Eria* in its former circumscription, or to split *Eria* into about 20 smaller genera, a few of which would be difficult to distinguish on morphology. In the end, the latter course prevailed in the team that carried out this work, and the resulting classification is here presented.

e-pollinaria. The orchid pollinaria collection at the Lankester Botanical Garden

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The diversity of Orchidaceae is in part the result of its complex zoophilous relationships and coevolutive adaptations with pollinators. Orchid pollen is frequently associated to other structures, forming functional units to facilitate its transport between flowers, called pollinaria. Pollinaria present a broad array of structures and shapes, whose morphology constitutes a tool for species identification, bringing information about the natural history of the orchid groups and helping in the reconstruction of evolutional lineage hypotheses. A direct comparison of pollinaria from previously identified species could greatly simplify species identification. However, pollinaria represent a huge challenge when it comes to being preserved, as they undergo severe distortions during dehydration, and are commonly affected by fungi that deteriorate the structures.

Before being stored in ultra-cold, pollinaria at the LBG are documented with high-resolution microphotographs, which provide information almost equivalent to that of fresh vouchers. Each series of images is associated with a technical sheet, including morphological description and measurements of pollinaria, allowing searches for specific morphological characters. A set of quality images, e-pollinaria, representing a broad spectrum on Neotropical orchid genera and species, will be soon provided through the net to researchers worldwide for potential application in various disciplines such as ecology, biology, entomology and paleobotany.

Towards a more inclusive concept of *Rhipidoglossum* Schltr.

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Recently-obtained phylogenetic trees have revealed that the Afrotropical angraecoid genus *Rhipidoglossum* was paraphyletic relative to *Cribbia*, *Margelliantha* and *Rhaesteria*. By integrating morphological evidence into the available molecular framework, we propose that *Cribbia*, *Margelliantha* and *Rhaesteria* should better be treated as part of *Rhipidoglossum*. Additionally, *Angraecopsis pusilla*, *Diaphananthe millarii* and *D. caffra* were also found to be embedded in *Rhipidoglossum*. Diagnostic features of this more inclusive monophyletic concept of *Rhipidoglossum* include the presence of pollinaria with two separate disk-shaped viscidia, a trilobed non-papillate rostellum, with its midlobe more prominent than the lateral lobes, and an undivided lip. In agreement with this new taxonomic treatment 11 new combinations are proposed. Following this revised generic concept, *Rhipidoglossum* now ranks among the most species-rich genera of angraecoid orchids, comprising approximately 50 to 60 species. Finally, future directions for further revisionary work of *Rhipidoglossum* and allied taxa are presented.

Integrative taxonomy of the section *Pseudophrys* (*Ophrys*, Orchidaceae): making use of both genetic and phenotypic data

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Accurate species delimitation is a prerequisite for most researches about biodiversity and its management. Integrative taxonomy has been advocated for a long time, yet tools allowing true integration of genetic and phenotypic data have been developed quite recently and applied to very few models. In particular, these tools have never been applied to orchids, despite many discussions about species delimitations in this family. In this study, we investigated species boundaries within a group of twelve *Pseudophrys* taxa by analyzing genetic, morphometric and chemical data in a Bayesian framework. We found that these twelve taxa were merged into four species when only genetic data were used, while most formally described species were recognized as such when only phenotypic data were used. The result of the IBPP analysis performed on both genetic, morphometric and chemical data supports the proposal to merge *Ophrys bilunulata* and *O. marmorata* on the one hand, and *O. funerea* and *O. zonata* on the other hand. We are convinced that this integrative taxonomic approach holds great promise to conduct taxonomic revisions in other orchid groups.

An interactive web-based key for two genera of necklace orchids from South East Asia

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We present an interactive web-based key to the 168 species of *Glomera* and *Glossorhyncha*, two genera within in the necklace orchids (Coelogyininae, Epidendroideae), 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. This online key is easier to use by novice users and also written in two languages, English and Bahasa Indonesia, and we hope therefore that anyone with an interest in wild orchids of Southeast Asia will contribute new observations to update current information on the distribution of these overlooked taxa.

Vegetative anatomy and micromorphology of *Neottia nidus-avis*

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Neottia nidus-avis, which is a saprophytic orchid, is generally distributed in shaded areas. It is usually represented by a single species and spread at north of Turkey. In our study, it was aimed to reveal the vegetative anatomy and micromorphology of *Neottia nidus-avis*.

The parts of the collected material were stored as herbarium sample, while other parts were preserved in alcohol as stock sample. During the definition of the species' anatomical features, the sections taken from roots, stems and leaves were analyzed using the Zeiss AxioLab A1 microscope and the Zeiss Axiocam 105 viewing system. The plant samples were mounted on stubs for scanning electron microscope by applying double-sided carbon tape. The mounted samples were coated with 12.5–15 nm gold–palladium (SEM coating system, SC7620). The analyses and scanning were performed on JEOL JMS-7001F Scanning Electron Microscope with a voltage of 5–15 kV.

The important characters in the anatomical examination of the root, stem and leaves are listed. In addition to the vegetative characteristics, micromorphological characters were determined.

This research was funded by a Grant from the Scientific and Technological Research Council of Turkey (TUBITAK, 114Z702).

Leaf anatomical and micromorphological characteristics of some Epidendroid (Orchidaceae) species

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Epidendroids are the largest group of Orchidaceae. This large group is spreading especially in tropical areas. But in the temperate zone, the numbers are too small to be understated. In this study leaf anatomical and micromorphological features of some Epidendroids species in the temperate zone have been revealed. Sections taken from the leaf for anatomical examinations were examined by light microscopy. While collecting anatomical sections, the location differences were considered, and for every feature, 30 measurements were taken in average using the same microscope. For micromorphological examinations, dried leaves were mounted on stubs using double-sided carbon tape and coated with 12.5–15 nm gold–palladium in a sputter coater. In our study *Cephalanthera*, *Limodorum*, *Listera* and *Neottia* were studied. As anatomical characteristics upper-lower surface cell, bulliform, stoma length-width and parenchymatic cell diameter for the leaf were analyzed. In addition, micromorphologically surface pattern and cell shape were examined. As a result of the examinations, similarities and differences among the genera have been revealed.

This research was funded by a Grant from the Scientific and Technological Research Council of Turkey (TUBITAK, 114Z702).

Comparative anatomy of root vascular bundles of some orchids

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Orchidaceae is the largest family of Asparagales and constitutes about 7% of all Angiosperms. The family, which has a wide spreading area on the world, problematic in terms of taxonomy due to similar flower structures and different applications. In our study, the structure of root vascular bundles of some Epidendroid and Orchidoid species was examined comparatively. Materials were collected from localities of Black Sea Region in Turkey. Samples for anatomical studies were fixed in 70 % alcohol. During the definition of the species root vascular bundles features, the sections taken from roots was analyzed using the Zeiss AxioLab A1 microscope and the Zeiss Axiocam 105 viewing system. While collecting anatomical sections, the location differences were considered, and for every feature, 30 measurements were made in average using the same microscope. In the study, root structures of some taxa belonging to 12 genera were examined in detail. In addition to reveal general anatomical features in the roots, the order of vascular bundles has been schematized. Comparisons were made between genera and taxonomic values of the root vascular characteristics were revealed.

This research was funded by a Grant from the Scientific and Technological Research Council of Turkey (TUBITAK, 114Z702).

Relationships of stomata size and genome size in Neottiae

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Abstract – Genome size is sometimes considered as an adaptive trait especially to environmental factors like climate or latitude. Relationships between cell size and DNA content has been reported for many species including plants. Cell size is also influenced by DNA endoreplication, which increases cell DNA amount. In plants, stomata guard cells exhibit some features including the lack of endoreplication. Consequently, guard cells are the most suitable cell type in plants to analyze such relationships. European genera and species of Neottiae exhibited variation of their 2C genome size. Guard cells have been thus measured in different Neottiae plants species. In *Epipactis*, several stands distributed in a limited geographic area and along an altitudinal gradient have been sampled. A general and positive significant relationship is thus obtained between DNA content and guard cell size within Neottiae. Variation among plants was less significant within *Epipactis* species. Only in *E. palustris*, a significant relationship was reported between DNA content and elevation. Relationships between cell size and genome size has been validated in Neottiae but not a general link to environmental conditions like elevation.

INTRODUCTION

Plants exhibit a large range of variation for genome size including among related species. This variation has been shown to be linked with the duration required for DNA replication and cell division rhythm and also to cell size (Cavalier-Smith, 1978). These cellular traits could be related to adaptation for environmental conditions. Consequently, genome size could be an adaptive trait mostly through variable amount of repeated sequences. The objectives of the study is thus to check relationships between cell size and DNA amount and then to test adaptive potential of genome size variation (to a large range of elevation). The study is carried out in Neottiae species that show a range of variation for 2C DNA content. Stomata are suitable to investigate cell size (no DNA endoreplication).

MATERIALS AND METHODS

Leaf collection: plant species listed in Table 1 (average of 5 plants per stand).

Cell size: measurement of length, width and area of stomata (Figure 1-a) with light microscope using Leica application suite (> 50 stomata per plant on lower leaf epidermis).

Variation among plants, stands and species have been analysed by hierachical analysis of variance with R.

Genome size is available for most Neottiae species (Prat *et al*., 2014).

Table 1. Species collected for measuring stomatal size.

Species	Number of stands
Cephalanthera damasonium	2
Cephalanthera longifolia	3
Epipactis atrorubens	9
Epipactis helleborine	5
Epipactis microphylla	1
Epipactis palustris	7
Epipactis placentina	1
Neottia ovata	1

RESULTS

Stomatal size showed a significant variation for all investigated parameters among plants, stands and species (*p-value* < 0.01).

Stomatal width and area are positively correlated with genome size (Table 2, Figure

2); the most significant relationships is for stomatal area and genome size, especially within genus *Epipactis*. Stomatal length, width and area are highly significantly correlated (Table 2).

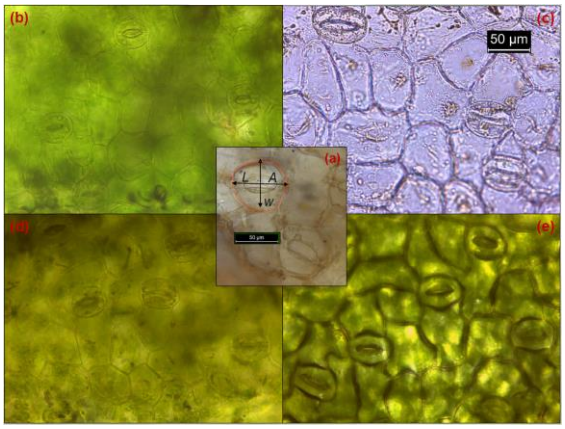

Figure 1. Lower epidermis of *Neottia nidus-avis* (a) with measured stomatal parameters (*L*: stomatal length; *w*: stomatal width; *A*: stomatal area), of *Cephalanthera longifolia* (b); of *Epipactis atrorubens* (c); of *E. helleborine* (d) and of *E. palustris* (e).

Stomatal size is not affected by elevation (Figure 3) in spite of a slightly negative relationships reported for *Epipactis* (r = -0.19 NS).

Table 2. Correlations in some Neottiae species between stomatal size and other traits.

	Stomatal length	Stomatal width	Stomatal area	Genome size
Stomatal width	0.66***			
Stomatal area	0.85***	0.95***		
Genome size	0.35	0.50**	0.51*	
Elevation	0.01	0.21	0.17	-0.01

Genome size and elevation are not related: the most positive relationship has been observed for *Epipactis palustris* (Figure 4).

CONCLUSION

Stomatal size varies according to plants, stands and species.

Genome size and stomatal size are significantly related in investigated species, particularly in *Epipactis* while *Cephalanthera* exhibited smaller stomata than expected according to their genome size.

Environmental factor, elevation in the present study, was not significantly related to stomatal size nor genome size. Only non significant trends have been observed.

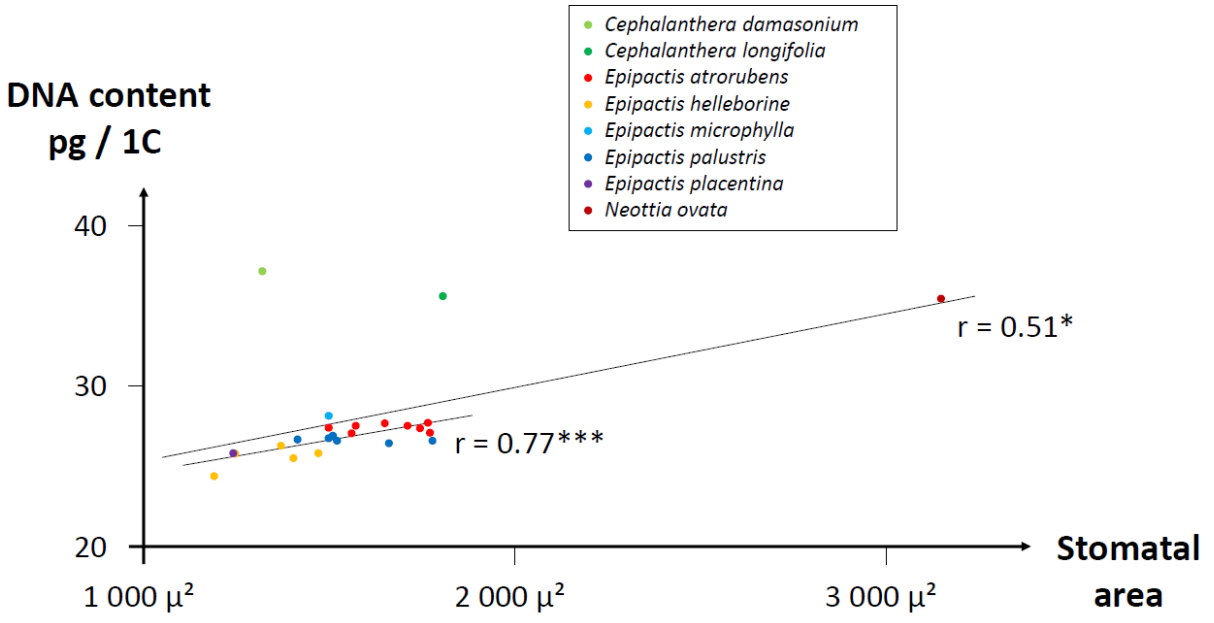

Figure 2. Relationship between stomatal area and genome size in Neottiae.

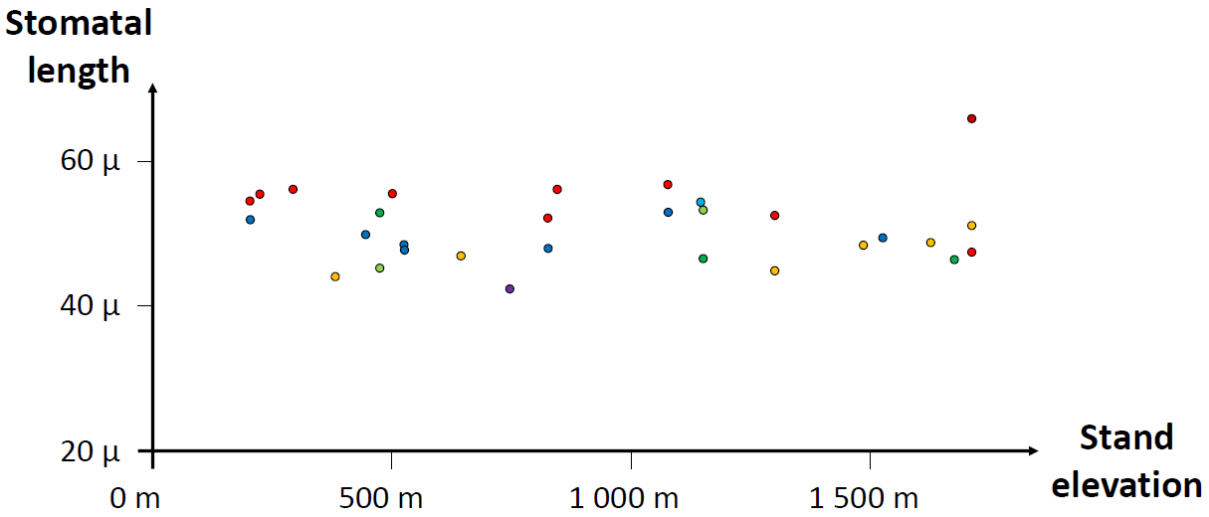

Figure 3. Relationship between stomatal length and stand elevation in Neottiae (see colour code in figure 2).

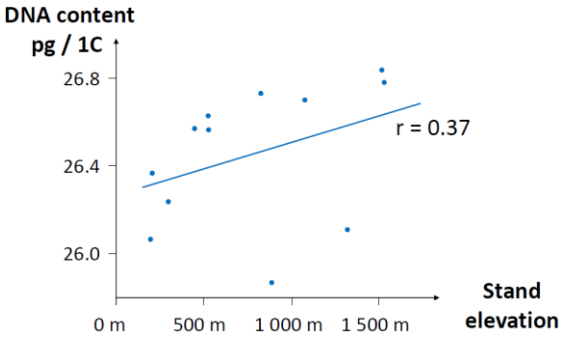

Figure 4. Relationship between genome size and elevation in *E. palustris.*

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Meiotic chromosome analysis in tropical orchid genus *Sobralia*

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Abstract – Tropical orchid genus *Sobralia* comprises terrestrial plants with elongated, cane-like stems and large symmetrical, yet ephemeral flowers. This genus is distributed throughout Central and South America and may hold horticultural potential. Nonetheless, little is known about the reproductive viability of species that comprise this genus. In this project male meiocytes have been examined by light microscopy to determine chromosome numbers, frequency of chromosome segregation defects and the frequency of normal tetrads at the end of meiosis II. The species sampled belong to an *ex situ* collection managed by the Lankester Botanical Garden of the University of Costa Rica. The species were: *S. amparoae, S. artropubescens, S. boucheri, S. bradeorum, S. carazoi, S. crispissima, S. danjanzenii, S. fenzliana, S. geminata, S. helleri* and *S. rosea.* Our results indicate that meiocytes from these species have a variable diploid (2n) chromosome number of 24, 30 and 32, that segregation defects are rare and that tetrad formation rates exceed 80%, suggesting efficient meiotic progression and high pollen viability. Taken together our results suggest that at least in Costa Rica the populations sampled are reproductively healthy and amenable to horticultural breeding.

Keywords: *Sobralia*, orchid, meiosis, chromosome analysis, Costa Rica.

INTRODUCTION

Little is known about how geographical isolation and the absence of pollinators impacts the reproduction of tropical orchids (Waterman and Bidartondo, 2008), Nonetheless it has been hypothesized that in Central America at any given time the number of fertile orchid plants is low, seed production is limited, and that therefore gene flow is also severely restricted (Tremblay and Ackerman, 2001). This set of conditions may lead to high selection pressures and the creation of new species (Tremblay *et al*., 2005).

To test these hypotheses experimental comparisons of populations are required, but usually this is not possible due to slow reproductive cycles, few flowers and little or no divergence in the genome of the individuals sampled (Lahaye *et al*., 2008). One approach to tackle these problems is to study chromosomes (Kao *et al*., 2007). Chromosomes are complexes of nucleic acids and proteins whose number and morphology varies across species thus allowing for evolutionary studies (Kao *et al.*, 2007; Lee *et al.*, 2011). Fortunately, the University of Costa Rica runs a dedicated orchid *ex situ* collection called the Lankester Botanical Garden. Within the garden a greenhouse is used to grow and study plants from orchid tribe *Sobraliae.*

Sobraliae is polyphyletic neotropical orchid tribe from the Americas that comprises about 200 species from genera *Elleanthus*, *Epilyna*, *Sertifera* and *Sobralia* (Neubig *et al*., 2011). These plants are often terrestrial plants with cane like stems and in the case of genus *Sobralia,* large flowers (Neubig *et al*., 2011). Flowers are symmetrical and beautiful, however little to no plant breeding has been performed in Costa Rica using *Sobralia,* a situation that makes little commercial sense considering the availability of native species, and its ease of cultivation and propagation, factors that have been determinant in the success of breeding programs elsewhere (Kamemoto and Kuehnle, 1996).

For this study *Sobralia* plants from *ex situ* collection of the Lankester Botanical Garden were sampled and pollinia were collected to determine the chromosome number and to observe and record chromosome segregation patterns during meiosis, including the formation of tetrads, which is the end stage of meiosis (Mercier *et al*., 2015).

MATERIALS AND METHODS

Pollinia from flower buds before anthesis were collected from the *ex situ* orchid collection located at the Lankester Botanical Garden, University of Costa Rica. Pollinia were processed according to Lee and Chung (2010). Briefly, they are placed in 2 mM 8-hydroquinoline (Sigma-Aldrich) solution for 5 hrs at 25° C, fixated in ethanol/glacial acetic acid solution (3:1, v/v) for 12 hrs and then frozen at -20° C. Samples were digested enzymatically with 6% cellulase and pectinase solution (Sigma-Aldrich) dissolved in 75 mM KCl at a pH of 4.0, for 1 hr at 37° C.

Digested samples were macerated in a drop of 40% acetic acid solution and then stained with fluorescent DNA stain 4′,6-diamidine-2′-phenylindole dihydrochloride (DAPI, Sigma-Aldrich). Images were obtained with a BX53 epifluorescence microscope (Olympus, Tokyo) connected to a ColorQ5 CCD camera (Olympus, Tokyo) and a Dell Precision Tower T7810 computer (Dell, Round Rock, TX). Images were analyzed with Adobe Photoshop CS5 (Adobe Systems, San José, CA).

RESULTS AND DISCUSSION

Analysis of meiotic cell cycle progression across all species suggested that there are no apparent defects during synapsis and pairing (zygotene and pachytene) (Mercier *et al*., 2015), and no defects during the alignment and segregation of bivalents (metaphase, anaphase and telophase I and II) (Mercier *et al*., 2015), as observed in Figure 1.

Counts of normal tetrads (regular tetrads) suggest that formation is normal (Table 1), suggesting normal chromosome segregation, and possibly formation of viable pollen after mitosis I and II. Preliminary chromosome counts during metaphase I and anaphase I suggest a variable chromosome number of 24, 30 and 32. Sampling will continue with frozen samples.

These preliminary results indicate that unlike in Puerto Rican *Lepanthes* (Tremblay and Ackerman, 2001), sexual viability in Costa Rican *Sobralia* is normal, suggesting reproductive success, effective population sizes, competitive ability, or ecological tolerance (Levin, 2002). For instance, sizeable groups of *Sobralias* are observed in disturbed habitats across Costa Rica (personal communication, Dr. Robert Dressler). It is also known that most *Sobralias* are pollinated by *Euglossine* bees and hummigbirds, which are common pollinators of Central American orchids (personal communication, Dr. Mario Blanco), and that these plants show synchronous gregarious flowering (Dressler, 1990). Therefore, availability of either a pollinator or a flowering partner does not appear to be a problem in these plants.

Table 1. Formation of tetrads is normal in *Sobralia* species, suggesting high pollen viability. Results are the mean of three biological and three technical samples, *n*=100 plus the standard deviation, nd: not determined.

Species	Normal Tetrads (%)	Chromosome number (2n)
S. amparoae	89,3±8,5	30
S. artropubescens	80,0±6,6	nd
S. boucheri	88,4±5,3	nd
S. bradeorum	89,7±1,8	nd
S. carazoi	85,7±5,1	nd
S. crispissima	83,1±10,2	nd
S. danjanzenii	93,5±0,7	32
S. fenzliana	87,5±1,0	nd
S. geminata	89,5±1,0	32
S. helleri	86,0±2,1	nd
S. rosea	93,1±2,1	24

Our results also indicate that wild individuals of *Sobralia* may be amenable to hybridization and plant breeding. Work is scheduled to continue until 2019 and may involve propagation by tissue culture.

Acknowledgements

This work was possible thanks to grant B5A49 from the office of the Vice Chancellor for Research and a travel permit to attend the EEOCE Paris 2018 from the Chancellor of the University of Costa Rica. The authors would like to thank the following students for their contribution to this project: Y. Corrales, M.A. Vega, K. Scott, K. Scott, and S. Castro-Pacheco. The authors also thank the scientific

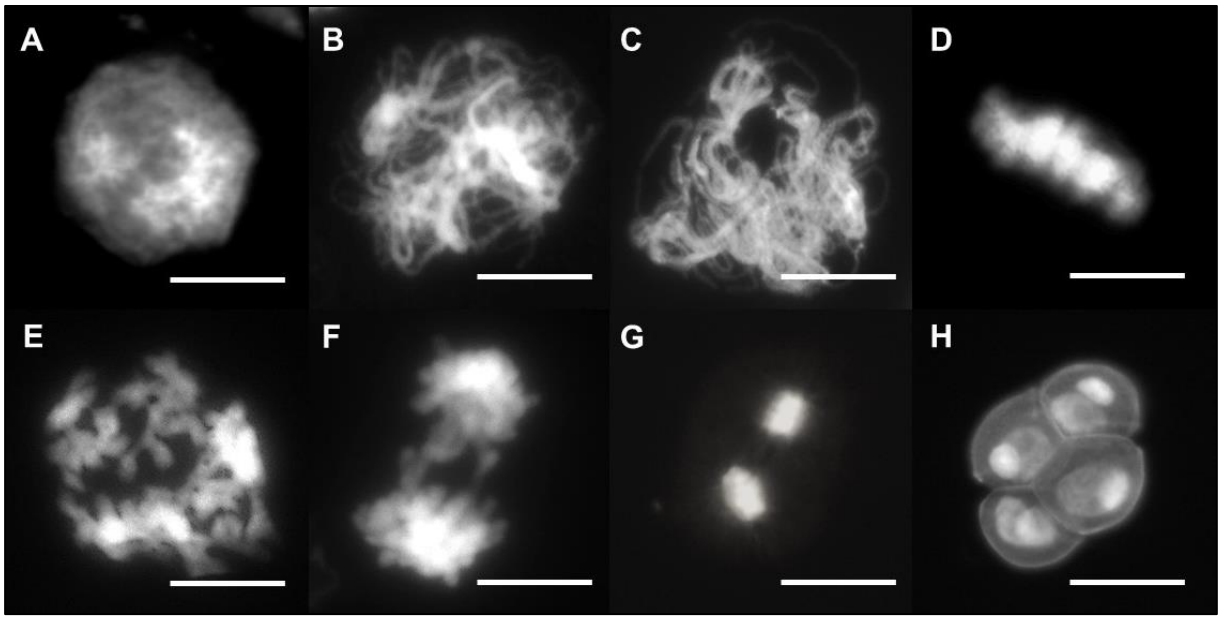

Figure 1. Cell division during male meiosis is normal in *Sobralia* species. Image is a representative composite from meiocytes across different species. A, leptotene; B, zygotene, C, pachytene; D, metaphase I; E, anaphase I; F, late anaphase I/early telophase I; G, metaphase II, H, tetrad stage. Scale bars, A-C, 20 µm, D-H, 10 µm. Meiocytes were stained with DAPI.

personnel of the Lankester Botanical Garden including Robert Dressler, Mario Blanco, Franco Pupulin and Jorge Warner.

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Pericarp anatomy and utrastructure of some Epidendroid (Orchidaceae) species

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Fruits have been largely ignored in orchid researches. In this study, we have investigated the anatomical, morphometrical and micromorphological properties of the pericarp to determine important diagnostic characters of the ovary and fruit belonging to some Turkish Epidendroid species. Transversal sections were taken to determine the anatomical and morphometrical characters for each taxon. In addition, micromorphological properties of pericarp surface were determined by electron microscopy studies. Various epidermal features such as cell shape, length and surface ornamentation were identified on pericarp. Moreover, secretory hairs having various size and shape were founded on fruit surface in *Cephalanthera rubra* and *Cephalanthera epipactoides*, *Neottia nidus-avis.* There were no striation and not visible cell border on fruit surface of *Limodorum abortivum*. There were also significant differences in terms of epidermal cell size. These findings have suggested that pericarp features could be diagnostic for epidendroid species.

This research was funded by a grant from the Scientific and Technological Research Council of Turkey (114Z702).

Flower arrangement in Southeast Asian orchids. Evolution trends and influence of environmental factors

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Abstract – The aim of our research is the comprehension of the organization and the characterization of Southeast Asian orchids. Until now we had not found complete and correct morphological description of flower arrangement in the orchid family. So it was not possible to understand the ways of their adaptation and evolutionary trends. Our research analyzed the flower arrangement and some parts of the androecium structure of more than 150 orchid species, belonging to 54 genera of Southeast Asia. The androecium micromorphology is helpful for the identification of the species and the study of the reproduction efficiency. The orchids of Southeast Asia show several inflorescence types, but often stop at the helicoid cyme stage of the ontogenesis. The inflorescence types can be modified by the environmental conditions in the wild or in cultivation.

Keywords

Environmental conditions, evolution trends, flower, inflorescence, ontogenesis, *Orchidaceae*, phylogeny, Southeast Asia.

INTRODUCTION

The *Orchidaceae* family has more than 25 000 species (Pridgeon *et al*., 2005). The flower arrangement of East Asian orchids has various types, which are formed either at the shoot top or in a lateral position (in the leaf axils), either with some bracts or without bracts, either by a single flower or by simple or complex inflorescences. The term inflorescence itself is controversial (Rudall and Bateman, 2010), regarding the limits of the flowers and their arrangement. In general, botanists drew attention to the modification of the inflorescence structure in orchids, but they compared the flower arrangement only for adult plants. Consequently, the description of the inflorescence is given with only two types: a raceme or a panicle (Wu *et al.*, 2009). However, racemes and panicles belong to the group of indeterminate inflorescences, and according to our observations, other inflorescence types are formed frequently in the *Orchidaceae* family, like the helicoid cyme, which is a determinate inflorescence. Another example of a determinate inflorescence is the terminal flower, which is formed not only on the tips, but also in the lateral leaf axis. In different environmental conditions a same species can show both inflorescence types or one of them. This raised doubts about the advisability of opposing a determinate and an indeterminate inflorescence (Parkin, 1914; Ricket, 1944).

In a number of cases, during the morphogenesis of the orchid inflorescence, a single flower, then a simple and a compound monochasium (determinate inflorescences) precede the formation of a raceme (indeterminate inflorescence). The genetic conditionality of the formation of indeterminate and definite inflorescences (Coen, 1991; Coen *et al.*, 1990, 1991; Coen and Meyerowitz, 1991, Coen and Nugent, 1994) could not be obtained. It was also not possible to experimentally change the structure of an indeterminate inflorescence (racemose) to a determinate (cymose). An attempt by

Baumann *et al*. (2015) to change the expression of *TFL1-2* and *tfl1-2* genes, was unsuccessful.

Given this situation, it was necessary to better study the ontogenesis stages, inflorescence morphogenesis and phylogenetic relationships of orchids. We have focused our research on the analysis of the morphological variability in the flower arrangement of Southeast Asian orchids, taking into account the morphogenetic, phylogenetic and geographic data.

MATERIALS AND METHODS

The results of the current study were first obtained from orchid material observed and collected during several expeditions in East Asia from 1998 to 2016 (Telepova-Texier, 2014; Telepova-Texier *et al.,* 2017). Then we have analyzed the transformation of the inflorescence structure during morphogenesis, mainly inside greenhouses of different botanical gardens (Muséum national d'Histoire naturelle, Paris, France; Komarov Botanical Institute, St-Petersburg, Russia; Botanical Garden-Institute, Vladivostok, Russia; Royal Botanical Gardens Kew, London, UK; Munich Botanical Garden, Germany; Botanical Garden of the University of Zurich, Switzerland). So, a comparison of the flower arrangement for both wild and cultivated orchid specimens could have been made.

We have analyzed 150 species belonging to four orchid subfamilies: *Cypripedioideae*, *Epidendroideae*, *Orchidoideae* and *Vanilloideae* (see Annex 1). Additionnally to the classical characteristics of the plant morphology, the androecium microstructure has been considered for the taxonomic identification of the studied species (anther caps and pollen dispersion units) (Telepova-Texier, 2017).

The identification of the inflorescence types was carried out on the basis of the classification proposed by Eichler (1875) which, despite the selection of determinate and indeterminate inflorescences, believed that in nature there are no sharp boundaries and one form can pass into another. The identification of complex and composite inflorescences was carried out proceeding from the structure of inflorescences duplicated on lateral axes (Fedorov and Artyushenko, 1979).

It is important to know that, the formation of a terminal flower ceases the growth of the inflorescence terminal axis, but it can continue to produce the lateral axes in space (Figure 1B). Not forgetting that the lateral axis has continued to develop also in the same time (Figure 1 A-B). When analyzing the arrangement of flowers, we have differentiated their location depending on the level of branching of the shoots (Kharchenko, 2012b). The structure of the shoot changes according to the principle of the decreasing proportion proposed by Troll (1969), for whom lateral shoots repeat the structure of the main shoot on a reduced scale.

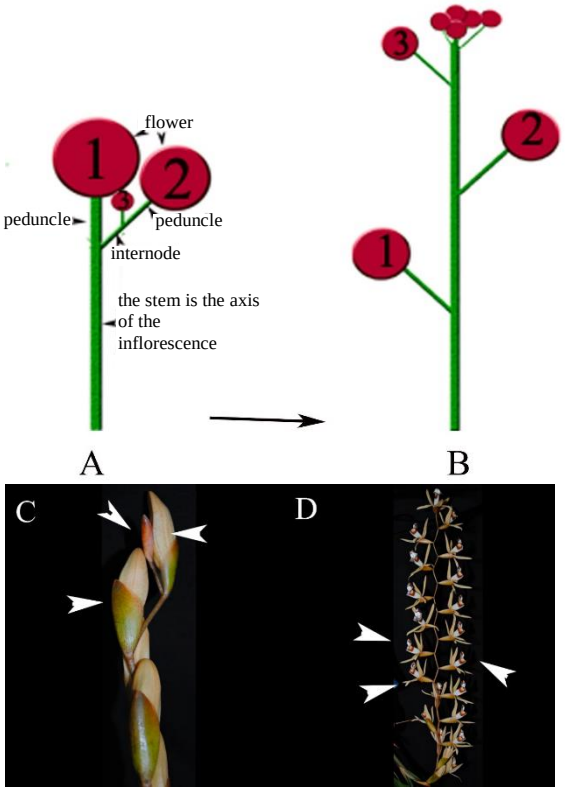

Figure 1. General scheme of formation of the floral axis during the morphogenesis of the inflorescence (1-3: order of flower apparition). Example of *Coelogyne massangeana* (C, D); A, C: formation of a compound monochasium (helicoid cyme); B, D: transformation of the helicoid cyme into a raceme.

For the analysis of the plesiomorphic and apomorphic state of the structure of the orchid inflorescences (Sherbakov and Kharchenko, 2018), cladograms have been constructed on

the nucleotide sequences of nuclear genes taken from the GenBank and constructed with the maximum likelihood method and the selected model of molecular evolution by means of the Mesquite system (Maddison and Maddison, 2018) and Iq-Tree (Nguyen *et al.*, 2014).

RESULTS

We have analyzed the inflorescence morphogenesis of 150 species from 54 genera belonging to four subfamilies of Southeast Asian orchids: *Cypripedioideae*, *Epidendroideae*, *Orchidoideae* and *Vanilloideae* (Annex 1, Table. 1 and Figure 2, Kharchenko, 2013, Telepova-Texier *et al.*, 2018).

Zygomorphic flowers of orchids can be single or several on the inflorescences. They can be formed on the tops of the shoots (*Cypripedium calceolus*, *C. macranthos*) or in the leaf axils (*Bulbophyllum macranthum*, *Dendrobium uniflorum*) (Table 1, Figure 2). Inflorescences can be with bracts or without bracts. Our research has shown that under greenhouse conditions, the number of flowers for each orchid inflorescence is significantly less than in nature (Kharchenko and Telepova-Texier, 2015). This is due to the effect of limiting factors, negatively affecting the productivity of plants (Telepova-Texier *et al*., 2016, 2018).

Seventy three % of the analyzed orchid species formed simple inflorescence (monochasium, compound monochasium, raceme, spike, head, umbel, spadix); less often (11% of species) they formed compound and complex inflorescences (helicoid cyme from helicoid cymes, helicoid cyme from racemes with terminal flower). Furthermore, single flowers developed in 15% of the species (Table 1, Figure 5). The study of the inflorescence morphogenesis in the four subfamilies of orchids indicates the presence of a general sequence of stages, that was limited in different species at different steps of formation (Table 1, Figure 2).

Some examples are provided here:

1. Representatives of subfam. *Cypripedioideae* have usually one terminal flower (*Cypripedium calceolus*, *C. macranthos*, *Paphiopedilum appletonianum*, *P. callosum*) or two terminal flowers (monochasium). So, their morphogenesis is composed of two stages (Figure. 2: 1-3, Figure 3: 1-2). In New Guinea, in a warm and humid climate, *Paphiopedilum rothschildianum* has a long flowering period, during which not 2 but 12 flowers are formed, which leads to the formation of a helicoid cyme (Figure 3: 4).

Terminal flower → Monochasium → Helicoid cyme.

2. In the subfam. *Vanilloideae*, in contrast to subfam. *Cypripedioideae*, a raceme is usually formed (*Vanilla annamica*, *V. siamensis*) (Figure 2: 1-4). However, in *Vanilla somae,* distributed to the north of China (with less favorable environmental conditions), usually there are only two flowers. Thus, in this case the morphogenesis ceases at an earlier stage (monochasium). So, the morphogenesis in *Vanilla* is represented by four stages:

Terminal flower → Monochasium → (Helicoid cyme) → Raceme with terminal flower.

3. Among the species of the subfam. *Orchidoideae*, *Epipactis helleborine*, which is a very common species in all European countries, has a raceme. For this reason, it is usually thought that the predominant type of inflorescence for this subfamily is the raceme. However, for the species of *Orchidoideae* studied in the tropics, we can observe other types of inflorescences, corresponding to earlier stages of ontogenesis:

Terminal flower → Monochasium → Helicoid cyme →Raceme with terminal flower.

The maximum duration of morphogenesis was found for *Ludisia discolor*, *Habenaria rhodocheila*, *H. medusa* and *Zeuxine flava*. Unlike most tropical orchids, that develop on trees and have inflorescences hanging down, these species are lithophytic or terrestrial, and their vertical inflorescences are oriented upwards. As the number of flowers increases, the axis of the inflorescence is straightened and the helicoid cyme is transformed into a raceme with a terminal flower (Figure 2:1-4).

4. In the subfam. *Epidendroideae*, a significant polymorphism is observed (especially in the genus *Bulbophyllum*), which is caused by a variability in the degree of axis

development (Figure 2: 1-4, Figure 3: 3, Figure 4: 2-6). The maximum time of morphogenesis course involves also 4 stages, the final one being the raceme (*Calanthe triplicata*, *C. cardioglossa*, *Pholidota ventricosa*, *Thelasis carinata*, *T. pygmaea*):

Table 1. Characters of the inflorescences and the androecium of the studied genera.

Genus	**Terminal flower**	**Monochasium**	**Helicoid cyme**	**Raceme**	**Spike**	**Helicoid cyme from helicoid cymes**	**Head**	**Umbel**	**Spadix**	**Axillary flower**	**Single flower**	**Simple inflorescence**	**Compound inflorescence**	**Aggregate inflorescence**	**One group of fertile anthers**	**PDU: sectile pollinia**	**PDU: Monads**
Acampe	-	-	+	-	-	-	+	-	-	-	-	+	-	+	+	-	-
Acriopsis	-	-	-	-	-	+	-	-	-	-	-	-	+	+	+	-	-
Aerides	-	-	+	-	-	-	-	-	-	-	-	+	-	-	+	-	-
Appendicula	-	-	+	-	-	-	-	-	-	-	-	+	-	-	+	-	-
Arundina	-	-	+	-	-	-	-	-	-	-	-	+	-	-	+	-	-
Ascocentrum	-	-	+	+	-	-	-	-	-	-	-	+	-	-	+	-	-
Bletilla	-	-	+	-	-	-	-	-	-	-	-	+	-	-	+	-	-
Bulbophyllum	+	+	+	+	+	-	+	+	+	+	+	+	-	-	+	-	-
Calanthe	-	-	+	+	-	-	-	-	-	-	-	+	-	-	+	-	-
Chelonistele	-	-	+	-	-	-	-	-	-	-	+	+	-	-	+	-	-
Chiloschista	-	-	+	+	-	-	-	-	-	-	-	+	-	-	+	-	-
Cleisostoma	-	-	+	+	-	+	-	-	-	-	-	+	+	+	+	-	-
Coelogyne	+	+	+	+	-	-	-	-	-	-	+	+	-	-	+	-	-
Cymbidium	+	-	+	+	-	-	-	-	-	-	+	+	-	-	+	-	-
Cypripedium	+	+	-	-	-	-	-	-	-	-	+	+	-	-	-	-	+
Dendrobium	+	+	+	-	-	-	-	-	-	+	+	+	-	-	+	-	-
Dipodium	-	-	+	-	-	-	-	-	-	-	-	+	-	-	+	-	-
Doritis	-	-	+	-	-	-	-	-	-	-	-	+	-	-	+	-	-
Eria	+	+	+	-	+	-	-	-	-	-	+	+	-	-	+	-	-
Epipactis	-	-	-	+	-	-	-	-	-	-	-	+	-	-	+	+	-
Flickingeria	+	+	-	-	-	-	-	-	-	-	-	+	-	-	+	-	-
Gastrochilus	-	-	-	+	-	-	+	-	-	-	-	+	-	-	+	-	-
Geodorum	-	-	+	-	-	-	-	-	-	-	-	+	-	-	+	-	-
Habenaria	-	-	+	-	-	-	-	-	-	-	-	+	-	-	-	-	+
Ludisia	-	-	-	+	-	-	-	-	-	-	-	+	-	-	+	-	-
Luisia	+	+	-	-	-	-	+	-	-	-	+	+	-	-	+	-	-
Macropodanthus	-	-	+	-	-	-	-	-	-	-	-	+	-	-	+	-	-
Oberonia	-	-	-	-	-	-	-	-	+	-	+	+	-	-	+	-	-
Paphiopedilum	+	+	+	-	-	-	-	-	-	-	+	+	-	-	+	-	+

Genus	**Terminal flower**	**Monochasium**	**Helicoid cyme**	**Raceme**	**Spike**	**Helicoid cyme from helicoid cymes**	**Head**	**Umbel**	**Spadix**	**Axillary flower**	**Single flower**	**Simple inflorescence**	**Compound inflorescence**	**Aggregate inflorescence**	**One group of fertile anthers**	**PDU: sectile pollinia**	**PDU: Monads**
Pelatantheria	+	+	-	-	-	-	+	-	-	-	+	+	-	-	+	-	-
Phalaenopsis	+	+	+	-	-	+	-	-	-	-	+	+	+	-	-	-	-
Pholidota	-	-	+	+	-	-	-	-	-	-	-	+	-	-	+	-	-
Pleione	+	-	-	-	-	-	-	-		-	+	+	-	-	+	-	-
Polystachya	-	-	+	+	-	-	-	-	-	-	-	-	-	+	+	-	-
Porpax	+	-	-	-	-	-	-	-		-	+	+	-	-	+	-	-
Renanthera	-	-	+	-	-	+	-	-	-	-	-	-	+	-	+	-	-
Rhynchostylis	-	-	-	+	-	-	-	-	-	-	-	+	-	-	+	-	-
Robiquetia	-	-	-	+	-	-	-	-	-	-	-	+	-	-	+	-	-
Seidenfadenia	-	-	-	+	-	-	-	-	-	-	-	+	-	-	+	-	-
Schoenorchis	-	-	+	+	-	+	-	-	-	-	+	-	+	-	+	-	-
Smitinandia	-	-	+	+	-	-	-	-	-	-	-	+	-	+	+	-	-
Spathoglottis	-	-	+	-	-	-	-	-		-	-	+	-	-	+	-	-
Thelasis	-	-	-	+	-	-	-	-		-	-	+	-	-	+	-	-
Thrixspermum	-	-	+	-	-	-	-	-	-	-	-	+	-	-	+	-	-
Trias	+	-	-	-	-	-	-	-	-	-	+	-	-	-	+	-	-
Trichoglottis	+	+	-	-	-	-	-	-	-	+	+	+	-	-	+	-	-
Trichotosia	+	-	-	-	-	-	-	-	-	-	+	-	-	-	+	-	-
Trudelia	-	+	-	-	-	-	-	-	-	-	-	+	-	-	+	-	-
Vanda	-	-	+	-	-	-	-	-	-	-	-	+	+	-	+	-	-
Vanilla	-	+	-	+	-	-	-	-	-	-	-	+	-	-	+	-	+
Zeuxine	-	-	+	-	-	-	-	-	-	-	-	+	-	-	+	+	-

Terminal flower → Monochasium → Helicoid cyme → Raceme with terminal flower.

The structure of the lateral axis of the inflorescence is not straightened. In particular, the structure of the inflorescence is a sympodial branching, and can develop by overturning in the helicoid cyme (*Appendicula hexandra, A. reflexa, Arundina graminifolia, Calanthe sieboldii, C. vestita, Chelonistele sulphurea, Coelogyne flaccida, C. pallens, C. trinervis, C. virescens, Dendrobium affine, D. glomeratum, D. moschatum, D. porphyrochilum, Dipodium paludosum, Eria lasiopetala, E. tomentosa, E. javanica, Pholidota chinensis, P. pallida, P. imbricata, Spathoglottis affinis, S. pubescens)* (Figure 2: 1-3, Figure 6):

Terminal flower → Monochasium → Helicoid cyme.

In *Coelogyne lawrenceana, Flickingeria fimbriata, Dendrobium ellipsophyllum, D. draconis, D. hercoglossum, D. glomeratum, Eria biflora, E. lasiopetala, E. tomentosa,* at the base of the solitar terminal flower is formed another flower. As a result, the inflorescence morphogenesis is represented by two stages (Figure 2: 1-2, Figure 8):

Terminal flower → Monochasium.

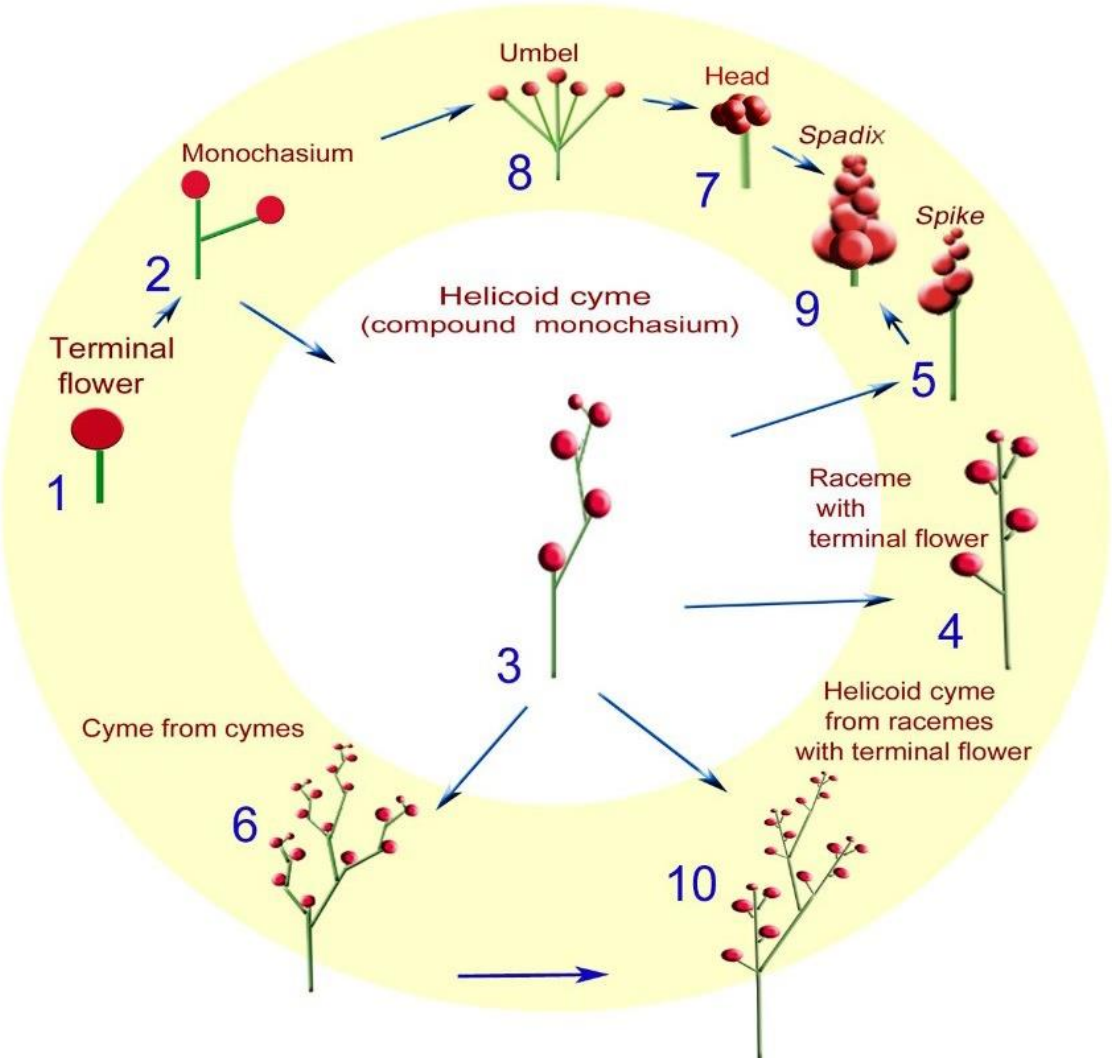

Figure 2. Evolution of the different types of inflorescences in Southeast Asian orchids. 1 - Terminal flower; 2 – Monochasium; 3 - Compound monochasium (helicoid cyme); 4 - Raceme; 5 – Spike; 6 - Compound monochasium from compound monochasium (helicoid cyme from helicoid cymes); 7 - Head; 8 – Umbel; 9 - Spadix; 10 - Aggregate inflorescence: compound monochasium from racemes (helicoid cyme from racemes).

In *Bulbophyllum lobbii*, *Eria lasiopetala*, *E. tomentosa*, *E. (Campanulorchis) thao*, *Pleione formosana*, *Bulbophyllum macranthum*, *Dendrobium uniflorum*, *Flickingeria xantholeuca*, *Porpax elwesii*, *Trias picta* and *Trichotosia velutina*, only one terminal flower is formed (Figure 2: 1, Figure 8):

Terminal flower.

In *Bulbophyllum lepidum* and *B. picturatum*, the internodes remain undeveloped, while the pedicels are well developed. As a result, an inflorescence of umbrella type (or fan) is formed (Figure 2: 1-2, 8, Figure 4: 2, Figure 5):

Terminal flower → Monochasium → Umbel.

In the case when the pedicels and internodes are poorly developed, as in *Bulbophyllum medusae* and *Eria globulifera*, the morphogenesis takes place in 3 stages, leading to a head inflorescence type (Figure 2: 1-2, 7, Figure 4: 3):

Terminal flower → Monochasium → Head.

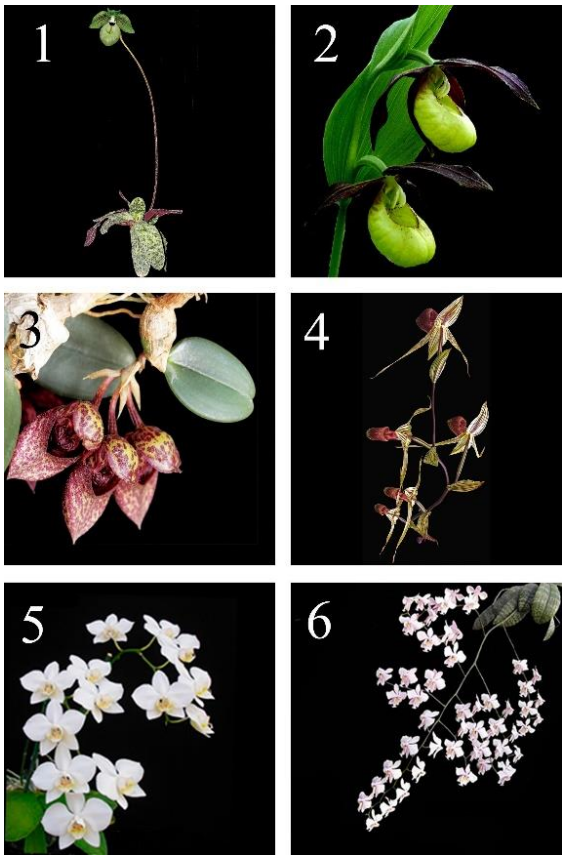

Figure 3. Inflorescence types of Southeast Asian orchids (part. 1). 1: *Paphiopedilum malipoense* - one terminal flower; 2: *Cypripedium calceolus* - monochasium; 3: *Bulbophyllum frostii* - compound monochasium; 4: *Paphiopedilum rothschildianum* - compound monochasium (helicoid cyme); 5: *Phalaenopsis amabilis* var. *aphrodite* - compound monochasium from compound monochasium (helicoid cyme from helicoid cyme); 6: *Phalaenopsis amabilis* var. *aphrodite* - aggregate inflorescence: compound monochasium from racemes (helicoid cyme from racemes).

However, if the axis of the inflorescence grows at the same time, then a cob can be formed as in *Bulbophyllum careyanum*, *Oberonia acaulis* and *Smitinandia micrantha* (Figure 2: 1-2, 9, Figure 4: 5):

Terminal flower → Monochasium → Spadix.

If, in the inflorescence, internodes develop and the flower have no pedicels, then a head or a spike is formed, like in *Bulbophyllum hirtum*, *Eria siamensis* and

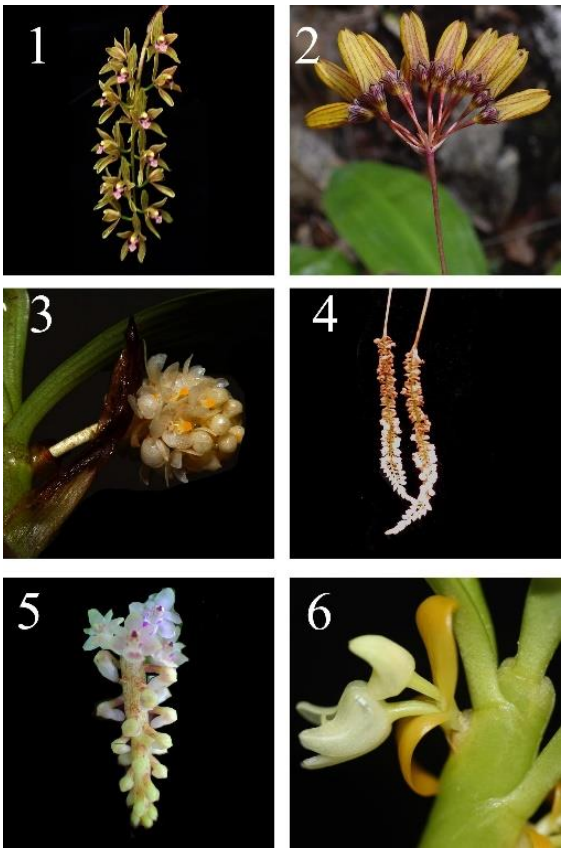

Figure 4. Inflorescence types of Southeast Asian orchids (part. 2). 1: *Cymbidium lowianum* - raceme; 2: *Bulbophyllum picturatum* - umbel; 3: *Eria globulifera* - head; 4: *Pholidota imbricata* - spike; 5: *Smitinandia micrantha* - spadix; 6: *Eria bifolia* - 2 lateral flowers.

Pholidota imbricata (Figure. 2: 1-2, 5, 7, Figure 4: 4):

Terminal flower → Monochasium → Head→ Spike.

In *Acriopsis javanica*, branching appears on the lateral axis of the inflorescence, with an increase of the flower number. Since the lateral inflorescences develop not in parallel, but in turn, it can be different stages of formation. Therefore, the morphogenesis of such an inflorescence can be represented as follows (Figure 2: 3, 6, 10):

Helicoid cyme → Helicoid cyme from monochasiums → Helicoid cyme from helicoid cymes → Helicoid cyme from racemes.

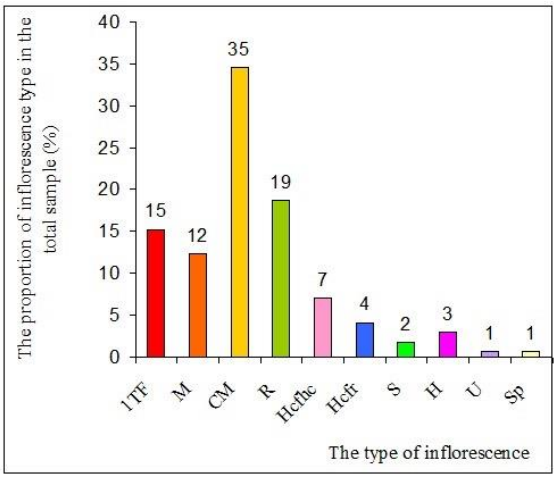

Figure 5. Proportion of inflorescence types (%) for the studied genera (see also Table. 1). One terminal flower (1TF); monochasium (M); compound monochasium (helicoid cyme) (CM); raceme (R); helicoid cyme from helicoid cymes (Hcfhc); helicoid cyme from racemes (Hcfr); spike (S); head (H); umbel (U); spadix (Sp).

Another species, *Acriopsis indica*, is more widespread to the north (India, Thailand, Vietnam) and at an altitude of 900 to 1800 meters. So, the development of its inflorescences ceases at an earlier stage of formation: Helicoid cyme from helicoid cymes (Figure 2: 1-2-3, 6).

5 In the representatives of subfam. *Epidendroideae* tribe *Vandeae*, the maximum duration of morphogenesis is observed in: *Cleisostoma arietinum, C. subulatum, Polystachya concreta, Schoenorchis gemmata* and *Smitinandia helferi* (Figure 2: 3, 6, 10):

Helicoid cyme → Helicoid cyme from monochasiums → Helicoid cyme from helicoid cymes → Helicoid cyme from racemes.

In plants with large flowers (4-10 cm, for example, in *Phalaenopsis amabilis*), the internodes are usually well pronounced, and thus a leafless inflorescence is formed:

Helicoid cyme or helicoid cyme from helicoid cymes.

In a number of species (*Acampe ochracea, Cleisostoma williamsonii, C. fuerstenbergianum, Renanthera imschootiana, R. monachica, Phalaenopsis amabilis, P. cornu-cervi, P. equestris, P. schilleriana, P. stuartiana* and *Vanda tricolor*), the morphogenesis of the inflorescence is limited at the following stage (Figure 2: 6, Figure 3: 5): Helicoid cyme from helicoid cymes.

In *Acampe praemorsa* and *A. rigida*, the internodes on the main axis are well developed, and on the lateral axis of the inflorescence they are short; therefore the structure of the inflorescences corresponds to the morphological definition of a "head". Accordingly, the morphogenesis involves the branching of the main axis of the inflorescence as a helicoid cyme, (Figure 2: 3, 6), and of the lateral axis respectively as a head (Figure 2: 1-2, 7):

Terminal flower → Monochasium → Head;
or:
Helicoid cyme → Helicoid cyme from monochasiums→ Helicoid cyme from head**.**

In those orchids, which form a lot of medium or large flowers (3-10 cm), such as *Aerides crassifolia, A. houlletiana, A. odorata, Cleisostoma simondii, Cymbidium atropurpureum, C. dayanum, C. lowianum, C. insigne, Doritis pulcherrima, Geodorum citrinum, Macropodanthus alatus, Thrixspermum amplexicaule, T. centipeda, Vanda lilacina, V. coerulea, V. brunnea* and *Phalaenopsis violacea*, the flowers are formed consecutively, and are grouped in simple inflorescences, with the following stages of morphogenesis (Figure 2: 1-3):

Terminal flower → Monochasium → Helicoid cyme.

The species *Pelatantheria bicuspidata* is widespread in China, Vietnam and Laos, at an altitude of 800-1400 m; it is distributed in rather harsh conditions and forms only 1-3 flowers, and its morphogenesis is usually limited at the stage of the monochasium (Figure 2: 1-2). The inflorescence morphogenesis is similar for *Trichoglottis pusilla, Trudelia alpina* and *T. cristata:*

Terminal flower → Monochasium.

Cymbidium goeringii is distributed in Northeast Asia. A short period of vegetation acts as a factor limiting the development of the

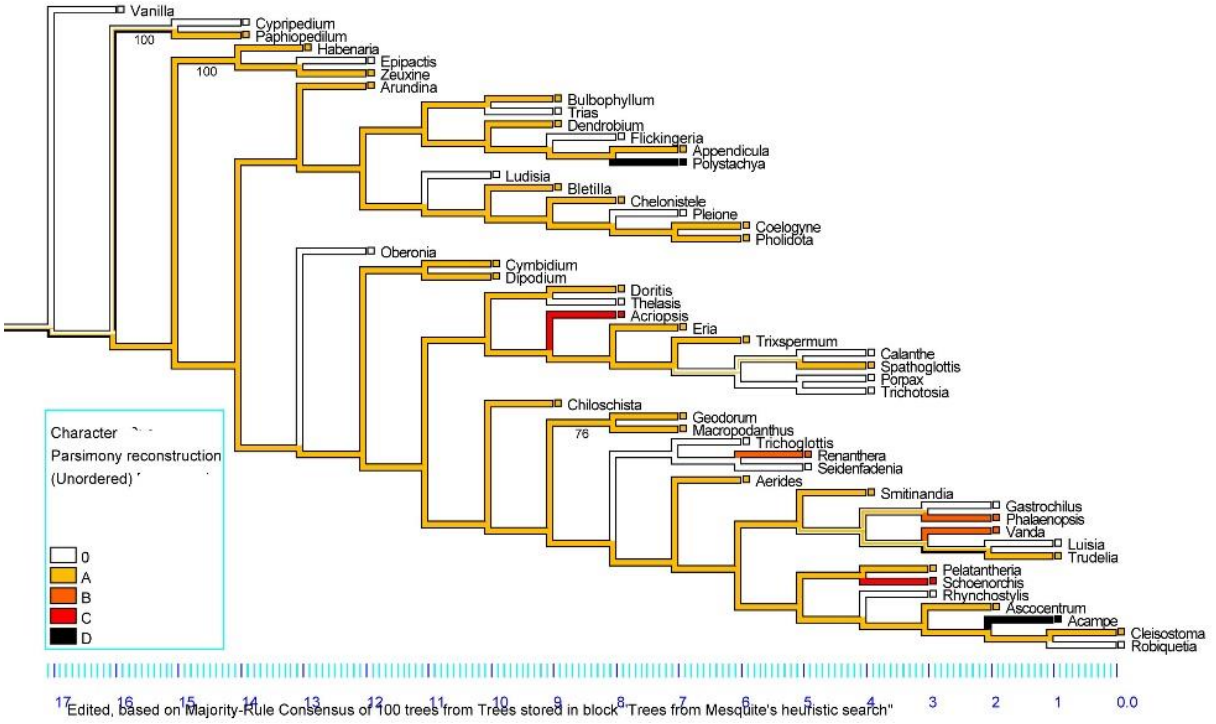

Figure 6. Cladogram showing the monochasium and raceme inflorescence types of the studied orchids. 0 : missing character; A: monochasium; B: monochasium and compound monochasium; C: monochasium, compound monochasium and raceme; D: compound monochasium and raceme.

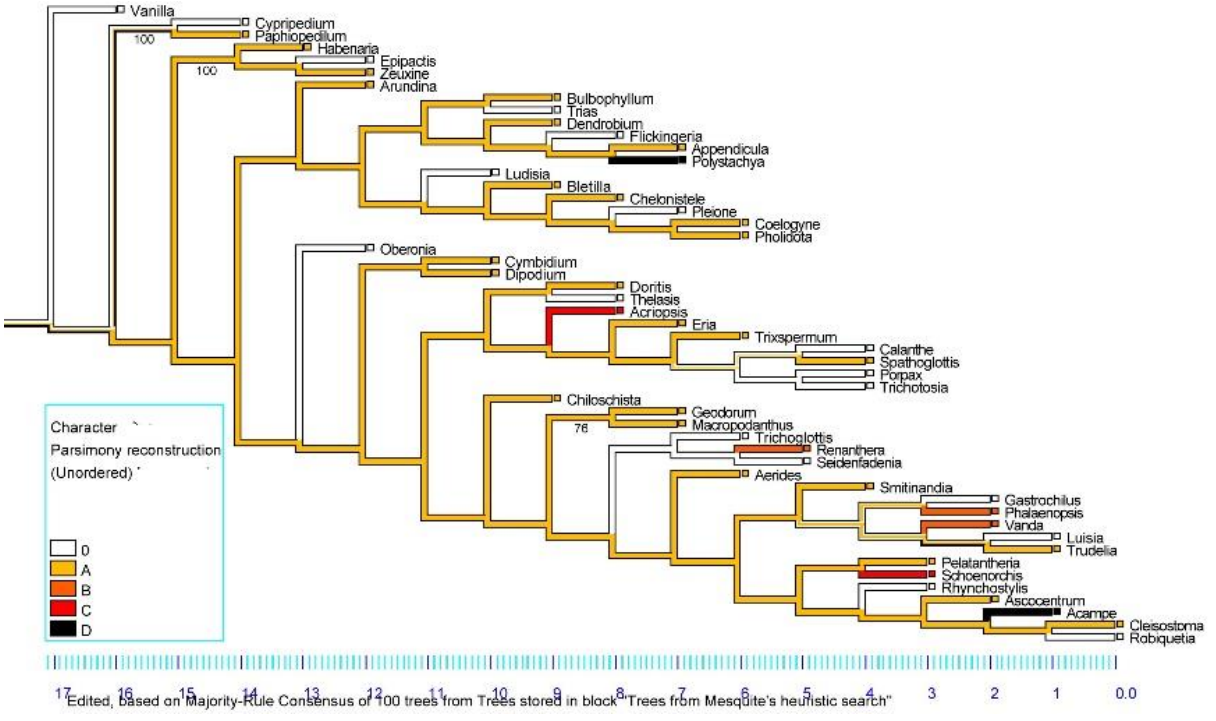

Figure 7. Cladogram showing the compound monochasium inflorescence types of the studied orchids. 0: missing character; A: compound monochasium (helicoid cymes); B: compound monochasium and compound monochasium from compound monochasiums (helicoid cyme from helicoid cymes); C, D: compound monochasium and compound monochasium from racemes (helicoid cyme from racemes).

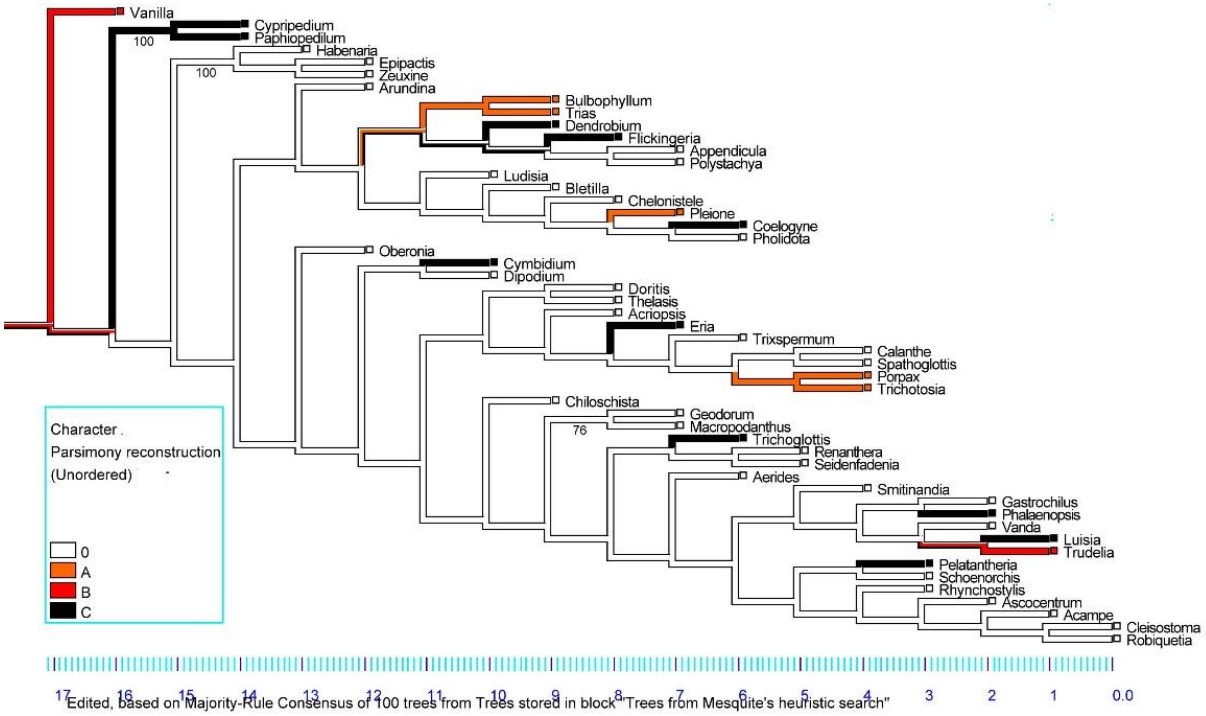

Figure 8. Cladogram showing the terminal flower and monochasium inflorescence types of the studied orchids. 0: missing character; A: terminal flower; B: monochasium; C: terminal flower and monochasium.

structure of the inflorescence for this species. Thus, a single flower is formed, due to the adaptation to harsh environmental conditions (apomorphy). In greenhouse conditions however, this species can form a monochasium.

Trichoglottis atropurpurea has a short vegetation period and forms a single terminal flower in the axils of the leaves.

In *Chiloschista usneoides*, *Cleisostoma discolor*, *Gastrochilus calceolaris*, *Luisia tristis*, *Rhynchostylis gigantea*, *R. retusa*, *Robiquetia minimiflora*, *R. spathulata*, *Schoenorchis fragrans*, *S. micrantha*, *Smitinandia micrantha* and *Seidenfadenia mitrata*, the morphogenesis involves the branching of the main axis of the inflorescence, with respectively, a helicoid cyme on the main axis and on lateral axis – a raceme (Figure 2: 3, 10):

Helicoid cyme → Helicoid cyme from helicoid cyme → Helicoid cyme from raceme.

In particular, *Luisia tristis* forms 3-10 small flowers (up to 2 cm), forming a raceme with terminal flower, but they open simultaneously. In the species *Luisia primulina*, *Pelatantheria insectifera* and *Gastrochilus obliquus*, the internodes of the inflorescences are poorly developed, therefore during the morphogenesis they can be traced to the following stages:

Terminal flower → Monochasium→ Head.

The studies carried out have shown, that the morphogenesis of the inflorescences of Southeast Asian orchids has different durations, and the formation of single terminal flowers in some cases is a plesiomorphic state, in particular in the subfamilies *Cypripedioideae* and also in *Epidendroideae*, and *Orchidoideae*, and apomorphic in *Vanilloideae* and in some species of *Epidendroideae* tribe *Vandeae* (ex. *Cymbidium goeringii*). On this basis, *Cypripedioideae* was used as the root group in a cladistic analysis of the morphology of flowers arrangement of Orchids (Figures 6 to 8).

We have noted that usually, in greenhouse conditions, the orchids of Southeast Asia have a terminal flower or its rudiment which dries up and remains underdeveloped at the end of the development of the inflorescence.

Therefore the identification of the inflorescence type depends on the conditions of cultivation, which predetermine the number of flowers and the stage of morphogenesis on which the development of the inflorescence ceases.

The results of our study have shown that the inflorescences of orchids have a general developmental sequence, and their polymorphism is caused by the restriction of the morphogenesis at different stages of formation. In the course of our analysis of orchid inflorescence diversity we can proposed the hypothesis of the heterochrony as a source of morphological transformation.

DISCUSSION

Based on the studies carried out, five types of morphogenesis of inflorescences have been identified for Southeast Asian orchids (Figure 2) :

1. Terminal flower → Monochasium → Compound monochasium (helicoid cyme) → Raceme with terminal flower;
2. Terminal flower → Monochasium → Helicoid cyme → Compound monochasium from compound monochasiums (helicoid cyme from helicoid cymes) → Compound monochasium from racemes (helicoid cyme from racemes);
3. Terminal flower → Monochasium → Umbel;
4. Terminal flower → Monochasium → Head → Spike → Spadix;
5. Helicoid cyme → Helicoid cyme from monochasiums → Helicoid cyme from helicoid cymes → Helicoid cyme from racemes.

1. Are determinate and indeterminate inflorescences fundamentally different in the course of their development?

At first, the study of the orchid morphogenesis indicates a gradual transformation of the inflorescence structure. A particular case of this is observed in *Cymbidium lowianum* (Figure 4: 1), where there is a transition from the helicoid cyme (determinate inflorescence) to the raceme (indeterminate inflorescence). Second, the analysis of the orchid phylogenesis have shown that the initial state for a raceme type is the helicoid cyme (Figure 7). Thus, we can observe the coincidence of changes in the inflorescence structure both in morphogenesis (ontogeny) and phylogenesis, which confirms the biogenetic law.

We want to draw attention to the fact, that changes in the inflorescence structure are quantitative characters. Therefore, determinate and indeterminate inflorescences can not be considered as fundamentally different in the course of their development. This also agrees with the opinion of many morphologists, in particular Parkin (1914) and Ricket (1944). They considered it inappropriate to contrast these types of inflorescences due to the fact that they can both be found on the same shoot and on plants of the same species that have grown up in different environmental conditions. That is, this variability is not inherited.

2. Does the structure of the orchid flower depends on its position in the inflorescence?

All orchids have zygomorphic flowers**,** which are usually grouped in determinate inflorescences: simple, complex or composite. The flowers have the same structure, regardless of whether if they are formed in the inflorescence with a terminal or a lateral position, on the main shoot or in the leaf axil (Figures 3 and 4). In addition, there are also single terminal flowers.

In the works of Carpenter and Coen (1990), Coen *et al*. (1990, 1991), Coen and Nugent (1994), Prusinkiewicz *et al*. (2007), the program for the development of inflorescence structure is redefined by the balance of the homeotic genes, in such a way that the lateral flowers are zygomorphic and form indefinite inflorescences, and the terminal flowers are actinomorphic, and form definite inflorescences. However, these conclusions are based not on the orchids, but on the cen and cyc mutants in *Antirrhinum majus* (Plantaginaceae) and the tfl mutants in *Arabidopsis thaliana* (Brassicaceae). The assumption that zygomorphic flowers are usually associated with indeterminate inflorescences, whereas actinomorphic flowers occur in determinate inflorescences, was also expressed by Stebbins (1973). The advisability of contrasting determinate and indeterminate inflorescences has been called into question since 1826, and the most famous morphologists participated in the discussion: Wydler **(**1851), Eichler (1875), Celakovsky (1893), Parkin (1914), Bentman (1918),

Goebel (1931), Ricket (1944) and Müller-Doblies **(**1977). In our works (Kharchenko, 2012a, Telepova-Texier *et al.*, 2016) the question of the affiliation of the inflorescences to this or that type is posed in connection with their individual or environmental variability in structure. In turn, Takhtajan (1964) paid attention to the fact, that it can be difficult to assign little-flowered inflorescences (2-3 flowers) to any of the types. For example: *Cypripedium calceolus* can formed 2-3 flowers, and we are not surprised that both its flowers are zygomorphic; while its inflorescences can be described as two different types (monochasium or compound monochasium).

Moreover, often only an approximate morphological characteristic of the inflorescence or the wrong one is given (Wu *et al.*, 2009), where the inflorescences of orchids are most often characterized as raceme or panicle type (indeterminate inflorescences), although, in some cases, they form determinate inflorescences, in particular: helicoid cyme (*Paphiopedilum rothschildianum*), helicoid cyme from helicoid cymes (*Phalaenopsis amabilis*) and helicoid cyme from racemes (*Acriopsis javanica*).

In the light of these results and discussions, we can therefore say that the structure of the flower do not depends on its position in the inflorescence.

3. Homeosis and heterochrony in the regulation of orchid inflorescence structure.

The assumption of Carpenter and Coen (1990) is based on the fact that, the homeosis and the heterochrony are identical in plants, unlike animals, due to the repeated formation of organs during the ontogeny. The meaning of homeostatic mutations consists in the changing of plan for individual development with a possible phylogenetic outlet (Korochkin, 2002), and heterochrony leads to a change in the time of the bookmarking of the organs (or the system of organs) in ontogenesis (Haeckel, 1866). It is difficult to imagine, that quite large-scale genetic changes, that change the plan of the structure of the reproductive system (the types of inflorescences), occurred independently and repeatedly in the evolution of Southeast Asian orchids (Figures 6-8).

Our analysis of the orchid morphogenesis has shown that the single terminal flower and the indeterminate inflorescences are the preceding stages in the formation of the raceme type; and the differences are not qualitative (depending on homeostatic genes), but quantitative and are a consequence of heterochrony (Karchenko, 2012a). Heterochrony can develops at different stages of the ontogeny (Severtsov, 1939), and is depending on the stage, where the development stopped. Severtsov distinguished three main modes of variability: the *anabolism* – a change in the final stage of the ontogenesis, the *deviation* - a change in the middle stages, and the *arhallaxis* - a change in the initial stage. For example, in the genus *Acampe*, the neoteny in the deviation stage can lead to underdevelopment of internodes and pedicels (Table 1). The changes in the morphogenesis of the inflorescence can be both positive and negative. Among the negative qualities of heterochrony we can mention the neoteny, suggesting a transition to the reproductive phase before the development of the adult body. In the opinion of Arber (1937) and Takhtajan (1970, 2009) however, the neoteny could be decisive not only in the adaptation of flowering plants, but also in their origin. Unfortunately, the genetic conditioning of neoteny has not been shown previously, despite the fact that Raff and Wray (1989) showed that the *TFL* gene has a heterochronic effect. This *TFL* gene can act as the coordinator of the ontogeny program as a whole, and its mutations lead to the neoteny (Kharchenko and Koksheeva, 2015).

Unlike Carpenter and Coen (1990), we consider it expedient to distinguish the homeosis and the heterochrony in plants as in animals. So, in our hypothesis, the heterochrony is thought to determine evolutionary trends in flower arrangement of Southeast Asian orchids. Within the genus *Cymbidium* for example, three types of inflorescences are observed and their occurrence may be due to the neoteny: single terminal flower, compound monochasium (helicoid cyme) and raceme (Table 1, Figure 2: 1-4, Figure 6). In particular, *Cymbidium lowianum*, which is distributed in Far East India (Figure 9), forms in nature 20-100 inflorescences (helicoid cymes), each composed of 12-40 flowers or more (Figure 4: 1):

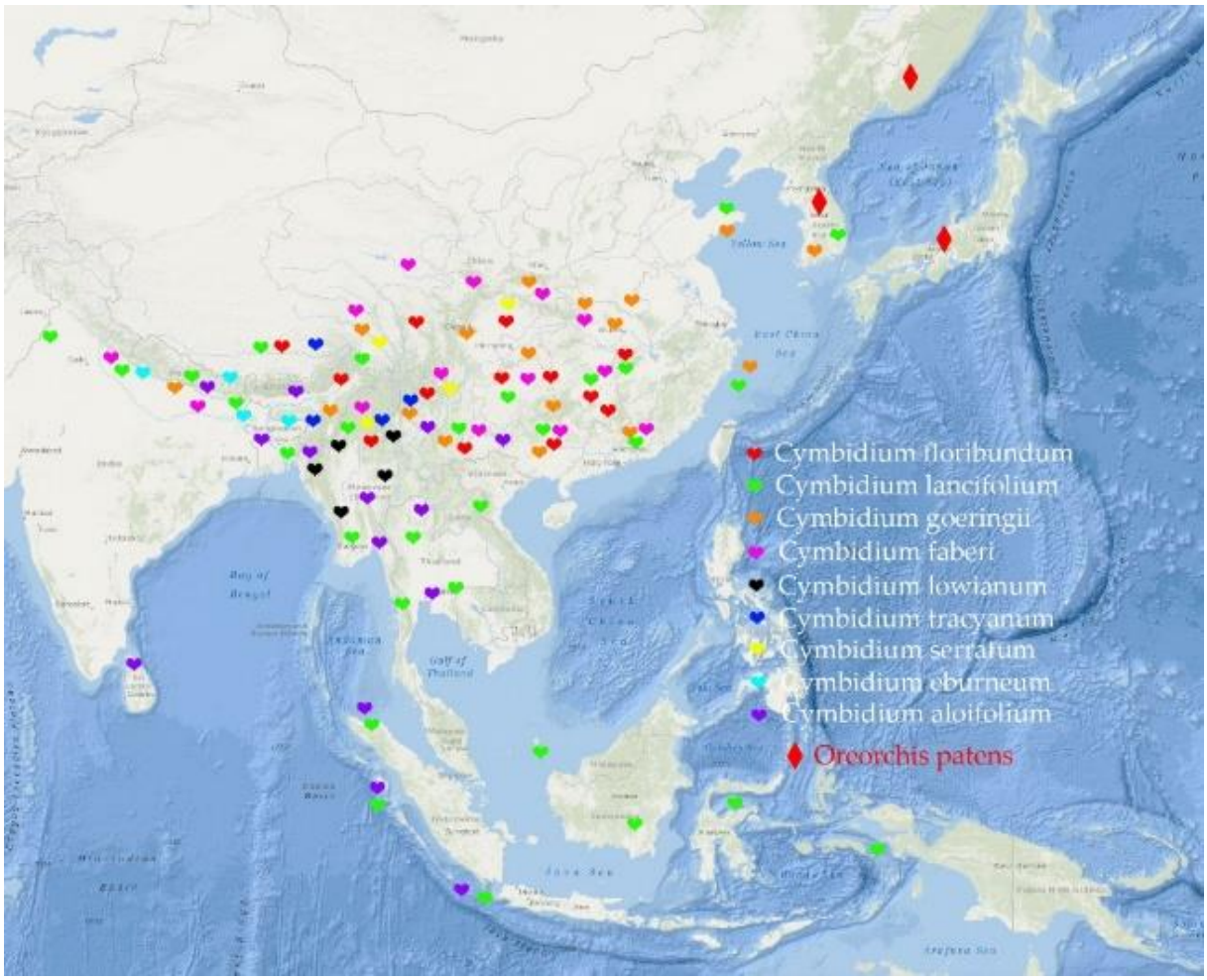

Figure 9. Distribution map of the studied *Cymbidium* and *Oreorchis* species.

Terminal flower → Monochasium → Compound monochasium (helicoid cyme) → Raceme.

Cymbidium goeringii is distributed in East Asia, until Japan, in the most northeastern distribution area of the genus *Cymbidium*, where it usually forms only one terminal flower. The neoteny in the arhallaxis stage can confine itself to the formation of only single flower. A monochasium can however be formed in more favourable environmental conditions (Table 1, Figure 2: 1-2):

Terminal flower → Monochasium.

Cymbidium dayanum, forms 20-30 flowers and grows in East temperate and tropical Asia (Table 1, Figure 2: 1-3), forming:

Terminal flower → Monochasium → Compound monochasium (helicoid cyme).

4. Evolutionary trends of flower arrangement in Southeast Asian orchids.

To determine evolutionary trends, it is important to identify the plesiomorphic and apomorphic state in the arrangement of flowers. Therefore, we have first constructed a cladogram for all the studied species of orchids of Southeast Asia on the basis of the comparison of several nucleotide sequences of nuclear genes (Sherbakov and Kharchenko, 2018). Later on, based on it, a number of cladograms were obtained reflecting the sequence of evolutionary events during the formation of the inflorescences. Three cladograms are presented in this paper (Figures 6-8). The diversity in the flower arrangement is due to the stopping of the morphogenesis at an earlier stage of formation, or, on the contrary, the addition of new stages. This indicates the decisive role of heterochrony in the evolution of flower arrangement.

The monochasium is the plesiomorphic (initial) state for all types of flower arrangement (Figure 6), and the compound monochasium and the raceme are apomorphic in comparison. In this case, the compound monochasium is a plesiomorphic state with respect to the simple inflorescence (raceme) (Figure 6), the compound inflorescence (compound monochasium from compound monochasium or helicoid cyme from helicoid cymes) and the aggregate inflorescence (compound monochasium from racemes) (Figure 7). The presence of a terminal flower is an apomorphic state for orchids of Southeast Asia (Figure 8), despite the fact that its formation is the starting point for any type of inflorescence (Maresquelle, 1964, 1970; Maresquelle and Sell, 1965). Thus, in the phylogeny of the orchids of Southeast Asia, the structure of the inflorescence is repeatedly transformed like morphogenetic changes (Figures 6-8). The transition of a compound monochasium into racemes is quantitative and may occur in orchids under the influence of the environment, as well as the mutations. According to the opinion of Carpenter and Coen (1990), Coen *et al*., (1990, 1991), Coen and Nugent (1994), Prusinkiewicz *et al*. (2007), such a transition is a consequence of the action of homeotic genes, which should lead to fundamental changes in the program for the structure development and to the aromorphosis (Severtsov, 2008).

5. Does the geographic and environmental conditions of Southeast Asia affect the flower arrangement of orchids?

In his time, Eimer (1898) had expressed the idea of the source of the inflorescence diversity. In the course of the divergence of the species, he noted the differents stages of morphogenesis of the inflorescences, however there was no evidence of regulation of this mechanism.

Our study testify to the similarity of morphogenetic transformations, that lead to a sympodial type of the inflorescence growth in Southeast Asian orchids, where the helicoid cyme is most often formed (Table 1, Figures 5 and 7). In truth, the development of the inflorescence is limited at different stages of formation, but consists only of terminal flowers (Figure 1). Further and further from the species origin center, where the conditions favor the maximum or diversity, the variety of the forms is reduced, due to the increased influence of limiting factors (Mayr 1970). Therefore, the orchids, distributed at the edge of the range, usually have single flowers or their size is reduced (Figure 9). This is due to the fact that increasing the negative impact of limiting factors reduces their reproductive potential, *via* their productivity reduction, resulting in less flowers or their smaller size, which lowers the formation of pollen. Therefore, in the northern regions of Southeast Asia and in the mountains, the evolution often has progressed along the path of reducing the number of flowers up to one, as in the case of *Cymbidium goeringii* (Figure 5: 1, Figures 8-9), or to the formation of inflorescences with many flowers reduced to the minimum size (less than 1cm), as in the case of *Oreorchis patens* (Figure 9). We can therefore highlight the fundamental influence of the environment and geography on the formation of different types of orchid inflorescences, at least for the region of our study. Different conditions can favor one heterochronous mutation and eliminate others, contributing to the selection and divergence in the course of the speciation.

CONCLUSION

We have used reproductive abmodality in flower arrangements (particularly, the heterochrony in morphogenesis) to identify evolutionary trends and the speciation of Southeast Asian orchids. It appears that the source of species divergence is the stopping of inflorescence development to different stages of the morphogenesis. For better considering the formation of the inflorescence structure, we have relied on the fact that the differences between determinate and indeterminate inflorescences are not qualitative, but quantitative and are a consequence of heterochrony, rather than homeostasis.

The variants of the inflorescences development are as follows:

1. Terminal flower → Monochasium → Compound monochasium (helicoid cyme) → Raceme with terminal flower;

2. Terminal flower → Monochasium → Helicoid cyme → Compound monochasium from compound monochasiums (helicoid cyme from helicoid cymes) → Compound monochasium from racemes (helicoid cyme from racemes);

3. Terminal flower → Monochasium → Umbel;

4. Terminal flower → Monochasium → Head → Spike → Spadix;

5. Helicoid cyme → Helicoid cyme from monochasiums → Helicoid cyme from helicoid cymes → Helicoid cyme from racemes.

We have made a review of the evolutionary history of Southeast Asian orchids, taking into account their molecular data, and coordinating them with morphological structure of inflorescence types and some androecium characters. The results of our study will be useful for reconstructing evolutionary history of orchids in connection with their high morphological flower diversity and the inflorescence arrangements. A larger and more detailed analysis will be presented in our further works.

Aknowledgements

We express our sincere gratitude to Dmitry Yurievich Sherbakov, specialist in Molecular Biology (Head of the Laboratory of Genetic Systematics of the Limnological Institute of the Siberian Branch of the Russian Academy of Sciences, Irkutsk, Russia) for his advices and for his assistance in the processing of the orchid material selected from GenBank and the construction of cladograms from the studied orchid species for their inflorescence types and morphogenesis. We are also grateful to the staff of the Electron Microscopy Technical Plaform and to Eric Joly, Head of the Executive Directorate of Museums, Botanic Gardens and Zoos of the Muséum national d'Histoire naturelle, Paris.

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Annex 1. List of the 150 studied orchids from Southeast Asia

Acampe Lindl. (*A. hulae* Telepova, *A. ochracea* (Lindl.) Hochr., *A. praemorsa* (Roxb.) Blatt. & McCann syn. *A. papillosa*, *A. rigida* (Buch.-Ham. ex Sm.) P.F. Hunt)
Acriopsis Blume (*A. indica* C.Wright, *A. liliifolia* Reinwardt ex Blume syn. *A. javanica*)
Aerides Lour. (*A. crassifolia* C.S.P. Parish ex Burb., *A. falcata* Lindl. & Paxton, *A. houlletiana* Rchb.f., *A. odorata* Lour.)
Appendicula Blume (*A. hexandra* (J.König) J.J.Sm., *A. reflexa* Blume)
Arundina Blume (*A. graminifolia* (D.Don) Hochr.)
Ascocentrum (J.J.Sm.) Schltr. (*A. ampullaceum* (Roxb.) Schltr., *A. miniatum* Lindl.)
Bletilla Rchb. f. (*B. striata* (Thunb.) Rchb.)
Bulbophyllum Thouars syn. *Cyrrhopetalum* Thouars (*B. careyanum* (Hook.) Spreng., *B. frostii* Summerh., *B. hirtum* (Sm.) Lindl., *B. lepidum* (Blume) J. J. Sm., *B. lobbii* Lindl., *B. medusae* (Lindl.) Rchb., *B. macranthum* Lindl.)
Calanthe R.Br. (*C. cardioglossa* Guill., *C. sieboldii* Regel, *C. succedanea* Guillaum., *C. triplicata* (Willemet) Ames, *C. vestita* Lindl.)
Chelonistele Kraenzl. (*C. sulphurea* (Blume) Pfitzer)
Chiloschista Lindl., *C. lunifera* Rchb.f. (J.J.Sm.), *C. usneoides* (D.Don) Lindl.)
Cleisostoma Blume (*C. arietinum* (Rchb.f.) Garay, *C. birmanicum* (Schltr.) Garay, *C. discolor* Lindl., *C. fuerstenbergianum* Kranzl.), *C. racemiferum* (Lindl.) Garay, *C. rostratum* (Rchb.f.) Garay, *C. simondii* (Gagnep.) Seidenf., *C. subulatum* Blume, *C. williamsonii* (Rchb.f.) Garay)
Coelogyne Lindl. (*C. flaccida* Lindl., *C. lawrenceana* Rolfe, *C. pallens* Ridl., *C. trinervis* Lindl., *C. virescens* Lindl.)
Cymbidium Sw. (*C. aloifolium* (L.) Sw., *C. atropurpureum* (Lindl.) Rolfe, *C. chloranthum* Lindl., *C. dayanum* Rchb.f., *C. eburneum* Lindl., *C. ensifolium* (L.) Sw., *C. faberi* Rolfe , *C. floribundum* Lindl., *C. goeringii* Rchb.f., *C. insigne* Rolfe, *C. lancifolium* Hook., *C. lowianum* Rchb.f., *C. serratum* Schltr., *C. tracyanum* Rolfe)
Cypripedium L. (*C. calceolus* L., *C. macranthos* Sw.)
Dendrobium Sw. (*D. affine* (Decne.) Steud., *D. draconis* Rchb.f., *D. ellipsophyllum* T.Tang. & F.T.Wang, *D. glomeratum* Rolfe, *D. hercoglossum* Rchb.f., *D. moschatum* (Buch.-Ham.) Sw., *D. porphyrochilum* Lindl., *D. uniflorum* Griff.)
Dipodium R.Br. (*D. paludosum* (Griff.) Rchb.f.)
Doritis Lindl. (*D. boulbetii* Telepova, *D. pulcherrima* Lindl. syn. *Phalaenopsis pulcherrima* Lindl.)
Epipactis Zinn, (*E. helleborine* (L.) Crantz)
Eria Lindl. (*E. lasiopetala* (Willd.) Ormerod, *E. siamensis* Schltr., *E. tomentosa* (J.Koenig) Hook.f., *E. javanica* (Sw.) Blume, *E. biflora* Griff., *E. thao* Gagnep. syn. *Campanulorchis thao* (Gagnep.) S.C.Chen & J.J.Wood)
Eulophia Lindl. (*E. graminea* Lindl.)
Flickingeria A.D.Hawkes (*F. fimbriata* (Blume) A.D.Hawkes, *F. xantholeuca* Rchb.f.)
Gastrochilus D. Don (*G. obliquus* (Lindl.) Kuntze, *G. calceolaris* (Buch.-Ham. ex Sm.) D.Don);
Geodorum Jacks (*G. citrinum* Jacks)
Habenaria Willd. (*H. rhodocheila* Hance, *H. medusa* Kraenzl.)
Ludisia Rich. (*L. discolor* Ker Gawl. (A.Rich.)
Luisia Gaudich. (*L. tristis* (G.Forst.) Hook.f., *L. primulina* C.S.P.Parish & Rchb.f.)
Macropodanthus L.O.Will. (*M. alatus* (Holttum) Seidenf. & Garay)
Oberonia Lindl. (*O. acaulis* Hook.f., *O. rufilabris* Lindl.)
Oreorchis Lindl. (*O. patens* Lindl.)
Paphiopedilum Pfitzer. (*P. armeniacum* S.C.Chen & F.Y.Liu, *P. appletonianum* (Gower) Rolfe, *P. callosum* (Rchb.f.) Stein, *P. malipoense* S.C.Chen & Z.H.Tsi, *P. rothschildianum* (Rchb.f.) Stein)
Pelatantheria Ridl. (*P. bicuspidata* T.Tang. & F.T.Wang, *P. insectifera* (Rchb.f.) Ridl.)
Phalaenopsis Blume (*P. amabilis* (L.) Blume, *P. cornu-cervi* (Breda) Blume & Rchb.f., *P. equestris* (Schauer) Rchb.f., *P. lobbii* Rchb.f., *P. stuartiana* Rchb.f., *P. schilleriana* Rchb.f., *P. violacea* Witte)
Pholidota Lindl. (*P. chinensis* Lindl., *P. imbricata* Lindl., *P. pallida* Lindl., *P. ventricosa* Rchb.f.)

Pleione D. Don (*P. formosana* Hayata)
Podochilus Schltr.(*P. microphyllus* Lindl.)
Polystachya Hook. (*P. concreta* (Jacq.) Garay & Sweet)
Porpax Lindl. (*P. elwesii* (Rchb.f.) Rolfe)
Renanthera Lindl. (*R. imschootiana* Rolfe, *R. monachica* Ames)
Rhynchostylis Blume (*R. gigantea* Blume, *R. retusa* (L.) Blume)
Robiquetia Gaudich. (*R. minimiflora* (Hook.f.) Kocyan & Schuit., *R. spathulata* (Blume) J.J.Sm.)
Schoenorchis (Blume) Reinw. (*S. fragrans* (Parish & Rchb. f.) Seidenf. & Smitin., *S. gemmata* (Lindl.) J.J.Sm., *S. micrantha* Reinw.)
Seidenfadenia Garay (*S. mitrata* (Rchb.f) Garay)
Smitinandia Holttum (*S. helferi* (Hook.f.) Garay, *S. micrantha* (Lindl.) Holttum)
Spathoglottis Blume (*S. affinis* de Vriese, *S. eburnea* Gagnepain, *S. plicata* Blume, *S. pubescens* Lindl.)
Thelasis Blume (*T. carinata* Blume, *T. pygmaea* (Griffith.) Blume)
Thrixspermum Lour. (*T. amplexicaule* (Blume) Rchb.f., *T. centipeda* Lour.)
Trias Lindl. (*T. picta* Parish & Rchb.f.)
Trichoglottis Blume (*T. atropurpurea* Rchb.f., *T. pusilla* Teijsm. & Binn.)
Trichotosia Blume (*T. velutina* (Lodd. ex Lindl.) Kraenzl.)
Trudelia Garay (*T. alpina* (Lindl.) Garay, *T. cristata* Lindl.)
Vanda (R.Br.) Jones (*V. tricolor* Lindl., *V. lilacina* Teijsm. & Binn., *V. coerulescens* Griff., *V. brunnea* Rchb.f.)
Vanilla Mill. (*V. annamica* Gagnepain, *V. siamensis* Rolfe, *V. somae* Hayata)
Zeuxine Lindl. (*Z. flava* (Wall. ex Lindl.) Trimen syn *Z. parvifolia* (Ridl.) Seidenf.)

Differential tempo of flower shape evolution in Madagascan *Bulbophyllum* (Orchidaceae): first insights from 3D-microCT scanning and phylogenetic analyses

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Adaptive radiation is a key process underlying the origin of biological diversity. However, especially in plants, surprisingly little is known about how the tempo and mode of lineage diversification is linked to the evolution of phenotypic trait disparity. Inspired by animal speciation models, a common perspective is that morphological change in reproductive traits predominantly occurs during speciation rather than during earlier periods of a radiation. To test this hypothesis, we presently analyze the flower shape of Madagascan *Bulbophyllum* (c. 210 spp.) using 3D-microCT scanning and landmark analyses for comparison with a time-calibrated molecular phylogeny of this mid-to-late Miocene radiation (c. 10 Ma). Our survey of c. 40 species indicates that most of the analysed sections of the group (median stem ages c. 9.0–4.7 Ma) can be discriminated by flower shape, whereas a particular section (c. 22 spp.; crown age c. 3 Ma) also contains a few closely related species and sister pairs (c. 1.0–0.5 Ma) that strikingly differ in this trait. Overall, these preliminary results point at differential timings of trait evolution in this group, with major changes in flower morphospace being linked to initial diversification events rather than occurring predominantly during speciation viz. the later stages of this radiation.

Research of the diversity and distribution of the *Cattleya trianae* in ten regions, the department of Tolima – Colombia

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The largest number of orchid species in Colombia is concentrated in the Andean region. For this reason, it is the most studied area of the country. However, there is not a detailed study about the geographical distribution of the species and, in particular, of the *Cattleya* genus. Therefore, the goal of this research was to know aspects of the diversity and distribution of the *Cattleya trianae* in the department of Tolima - Colombia. Synthetic environmental profiles were build based on the ecological, climatic and environmental distribution conditions of *Cattleya trianae*. It was observed, a same species can be found under different altitude, temperature, soil type, precipitation, climate and evapotranspiration conditions. In this way, ten (10) regions were established where it is possible to find these species; six (6) *concolor* and one type varieties. It was observed, the species can possibly be found in a greater proportion in the central, north-eastern and south-eastern part of the department. In conclusion, it can be inferred that *Cattleya trianae* is a high adaptable species and it is geographical distributed in a strip that goes from the north-east to the south-east of the central part of the department.

Micromorphological characteristics of labellum and spur belonging to some *Orchis* and related genera

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A detailed morphometrical and micromorphological analysis of the labellum and spur were carried out using both light and scanning electron microscopy on species belonging to *Orchis* and related genera (*Anacamptis*, and *Neotinea*) of Orchidaceae in Turkey to identify diagnostic characteristics and to confirm whether there are features that are related to their pollination strategy. In the samples, various epidermal features such as shape and length of epidermal cell or papillary structures and surface ornamentation were identified on the adaxial surface of the spur and the labellum. In many species, characteristically shaped papillae were concentrated at the base part close to the gynostemium or the distal part of the labellum. Despite the abundance on the labellum surface in *Anacamptis laxiflora*, there was no papillary structure on all surfaces of the spur. In *Neotinea tridentata*, both labellum and spur surface were covered with convex cell, having no papillae. Furthermore, the labellum surface striation varied among the orchid genera. Our data enable us to distinguish between species and show congruence with the present circumscription of these related genera.

This research was funded by a grant from the Scientific and Technological Research Council of Turkey (114Z702).

Ecological constrains on ovule development in Mediterranean orchids

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Ovule development represents an important component of female reproductive investment in flowering plants. In orchids, ovule development and subsequent ovary enlargement is triggered by a signal produced by pollen arrival on the stigma (Zhang and O'Neill, 1993). This peculiarity, unique among angiosperms, is likely to confer an important resource allocation advantage in orchids as they typically produce thousands of ovules. Interestingly, the stage of ovule development before pollination has been found variable in different orchid groups ranging from ovule primordial to immature ovules (Tsai *et al.*, 2008). However, so far, it is not clear whether this variation is driven by a phylogenetic or an ecological constrain.

We investigated stages of ovule development at anthesis in the Mediterranean orchid genera *Anacamptis*, *Dactylorhiza*, *Himantoglossum*, *Ophrys*, and *Orchis*, all belonging to the monophyletic Orchidinae and characterized by different flowering times and pollination strategies (rewarding vs. deceptive).

We found that ovule development ranges from ovule primordial (with archesporial cells) to immature ovules (at first meiotic division with developed inner and outer ovule teguments). These results sharply contrast with what found in tropical orchids, as *Phalenopsis*, where ovule development is still at primordial stage even one month after pollination.

Variation in ovule development stages between closely related species was found depending on flowering time and pollination strategy. In one species pair, *Himantoglossum robertianum* and *H. hircinum*, a clear correspondence between flowering time and ovule development was observed. Indeed, at anthesis, in the early blooming *H. robertianum*, ovules are at the primordial stage, whilst in the late blooming *H. hircinum* inner and outer teguments are already developed. Likely *H. robertianum*, blooming in January, might benefit from a longer amount of time to complete ovule development before the annual summer drought. In another species pair, with contrasting pollination strategies, the rewarding *Anacamptis coriophora* and the deceptive *A. morio*, both flowering at the same time, we found that the rewarding species has developed ovule and teguments while *A. morio* ovules are still developing. Likely, *A. morio* may reduce the cost of advanced ovule development being exposed to the high risk of fruiting failure due to the low levels of pollination typical of deceptive species (Tremblay *et al.*, 2005).

Overall these results strongly suggest that, in the Mediterranean habitat, orchid ovule developmental stage is modulated by ecological factors, as flowering time or pollination strategy, and can be selected for optimizing the female reproductive investment.

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Tsai W.C., Hsiao Y.Y., Pan Z.J., Kuoh C.S., Chen W.H., Chen H.H. 2008. The role of ethylene in orchid ovule development. *Plant Sci.*, 175: 98–105.

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Variability in space and time: coexistence of contrasting fruit distribution patterns in *Orchis militaris*

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In flowering plants, a decrease of fruit production towards the apex of individual inflorescences is usually observed. Numerous studies have been conducted but variation among populations and years has rarely been investigated. In order to study geographical and temporal variation in fruit distribution pattern, fruit production was described in relation to flower position in *Orchis militaris.* During two years, eight populations were studied and fruit position along the inflorescence was recorded. A generalised linear mixed model analysis was performed to examine the effect of population, year, and relative flower position on fruit production. Four main patterns of fruit production were described: decrease towards the apex, increase towards the apex, higher fruit set in the middle part of the inflorescence associated with acropetal and basipetal decline, and uniform distribution pattern. Within a given population, patterns were either consistent or variable among years and the relationship between fruit production and flower position was not necessarily linear. Our study demonstrates the intraspecific diversity of fruit distribution patterns in *O. militaris*, and the necessity to include geographical and temporal variation in the sampling design. The behaviour of foraging pollinators and/or the temporal matching between floral phenology and pollinator's activity period may be responsible for several patterns.

Trehalose utilization in Orchidaceae family – unique among plants and conserved among orchids

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Trehalose is a common sugar widely distributed among almost all organisms. However, it's role in plants remains enigmatic. Most of the plant species including orchids possess extremely small concentration of trehalose. This sugar works as a signaling molecule in plants and is therefore not suitable as major energy and carbon source. In our research we studied extraordinary ability of orchids to utilize trehalose as the sole source of carbon and energy. The utilization of trehalose by orchids has been previously observed, however no comprehensive study has been made on this phenomenon. We performed *in vitro* asymbiotic cultivation experiments focused on influence of trehalose on germination, mortality, growth and endogenous saccharide content of orchids from different subfamilies. Compared to sucrose and glucose, trehalose is a similarly metabolizable and suitable source of carbon and energy for all selected orchid species. Analysis of sugar content in medium after cultivation supports hypothesis that trehalose is cleaved into glucose extracellularly. Furthermore, trehalose utilization can be inhibited by trehalase specific inhibitor validamycin A. Orchid ability to utilize trehalose is contrasting with proposed general inability of plants to utilize this sugar and might be a consequence of fungi-orchid coevolution.

Effect of a Mexican endemic orchid, *Prosthechea karwinskii*, on metabolic syndrome induced in Wistar rats

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Prosthechea karwinskii is an endemic Mexican orchid used as ornamental and in the traditional medicine to treat diabetes and some problems related with inflammatory processes.

To determine the antioxidant activity (AA) and to validate the medicinal use of this orchid using a rat model, the hydroalcoholic extract from this plant was evaluated for treat conditions relate to the metabolic syndrome. An *in vivo* assay 25 weaned male Wistar rats were divided into a control group (CG; n = 5) and a Metabolic Syndrome group (n = 20); the latter were induced to metabolic syndrome with 40% sucrose in the drink water for 13 weeks, then this group was subdivided into 4 groups: Metabolic Syndrome (MS, n = 5) received sucrose, and three groups receiving 200 mg/kg of body weight of pseudobulb (P, n = 5), leaf (L, n = 5), and flower (F, n = 5) extracts. All treatments were followed for 13 days; at the end blood was collected to measure glucose, cholesterol and triglycerides. AA were measured in the extracts by DPPH method. L extract had highest values in AA, followed by F and P extracts. L extract had highest reducing effect on glucose level too, while F extract had highest reducing effect on cholesterol and triglycerides levels. The extracts evaluated here reduced glycemic and lipidemic parameters in Wistar rats with MS induced. These effects may be attributed to its high AA.

Orchid judging at EOCCE 2018

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As in all major international exhibitions, a judgment has been held to reward the most beautiful plants presented. More than fifty judges from all over Europe (and even further afield, South Africa, Singapore) gathered in Paris. The judgment was organized by Clare Hermans and Vinciane Dumont, assisted by AFJO members, including Hélène Royer, Albert Falcinelli and David Lafarge.

In France, as in many countries, orchid shows are an opportunity to judge plants. Judging had ceased after the 18th World Orchid Conference in Dijon in 2005, but in 2011 a new training scheme was started. Judges are accredited after a three-year training program and then have two years probation. Three training centers are currently operational and are based in Bordeaux, Paris and Leucate. Judges are now fully qualified and belong to one of two associations. The AFJO (French Association for the Judgment of Orchids), is based in the south and EJO (School of Orchid Judges) works mainly in Île-de-France and in the northern part of the country. Members of both organizations regularly meet to judge together at shows.

The rules for plant and display judging are reproduced here from **The Judges Handbook.**

Display judging

All orchid displays will be judged for First, Second and Third place ribbons; EOCCE 2018 Trophies and Gold, Silver and Bronze Medals, a simple majority will determine any decision. All Ribbons and Medal cards will be put on the displays. Points for Medals: Bronze = 40-59 Silver = 60-79 Gold = 80-100 (Table 1).

Plant judging

All plants in the Show will be considered for First, Second and Third place ribbons in their class and for EOCCE 2018 Trophies (Table 2). Ribbons will be put with the awarded plant on the displays. All Registrants, who are Orchid Judges recognised by their national judging organization are invited to participate. Registrants, who are Trainee Judges, will be eligible for stewarding duties, Judges may also be asked to assist with stewarding. Judges must enrol before the 1st March 2018 (also see Rules). All Judges and Trainee Judges are also invited to participate in the European Judging Symposium on Friday afternoon.

Table 1. Pointing system for displays.

	Points
General impression (aesthetic, originality, colours, legibility, balance, etc.)	30
Range of orchids	20
Plant condition	20
Number of plants	20
Labelling, educational and nicely finished	10
Total	100

EOCCE 2018 Judging Guidelines

Cut flower spikes cannot be taken into account for ribbon judging but can be considered for Gold, Silver and Bronze Medals

Table 2. Pointing system for plants.

Culture	**Points**
General aspect	20
Health & condition	10
Number of flower	10
Flower	
Presentation	20
Colour	20
Size and shape	20
Total	100

at the discretion of the judges. However plants can only be considered for ribbon judging and not Medals.

Made-up plants (group of plants in one container), cannot be judged as a single plant but individual plant from such a group can be considered in their own right.

Although comparative judging is used, if no plant of adequate quality can be found for a place in any class it is permissible not to award that ribbon.

To make certain that the Judges enjoy a display in its full glory Exhibitors should make sure that all lighting, water-features etc. are switched on during judging of displays.

Stewards will normally be Trainee Judges and therefore have no voting rights, however some groups may have the benefit of a fully qualified Judge as Steward, in this case this person will have voting rights.

It is important that Judging Teams adhere to the time-table.

Early access to the show has been provided, so all Judges and Stewards should spend as much time as possible looking at plants before actual judging starts. After meeting the Judging Team should initially make an informal tour of the show with all members making note of those plants and cut flower spikes within the groups remit that catch the eye. At the end the Team should have arrived at an agreed list of noteworthy plants and cut flower spikes. A further circuit can then be made to examine these formally and agree on a shortlist. Final ribbons and medals should then easily be agreed. Finally any Trophies to be awarded can be agreed on.

Every plant and cut flower spikes should be examined carefully. It may become apparent that a plant or cut flower spikes which looked a winner from some distance is much less good when seen closely.

Team Leaders are there to moderate and inspire the Team and should only vote if the votes by the Team are tied.

Whatever method is actually used, it is vital that Teams keep accurate records of results. These should be written on the result sheets provided and signed by the Team Leader. The sheets should be passed to the Judging Committee as a batch. Team Leaders should ensure that ribbons and medal cards are correctly placed and that one of the Team's Stewards knows exactly which plants have been recommended for trophies so that placement of these awards can be accurate. Stewards should write the name of the plant and its class on the back of any ribbons.

All Team Leaders should partake in the judging of the Premier Plant Trophies.

Should any member of a Judging Team have an interest in a plant or cut flower spikes that might be judged by that Team then that member must declare that interest and remain aloof from any judging that may affect that orchid. If a Team Leader should be in this position the Team must agree a temporary Leader to take charge while the affected class is judged. If no suitable member of the Team can perform this function, a member of the Judging Committee can be coopted.

All discussion should be conducted courteously and democratically.

Where errors or confusion cause problems please refer to a member of the Judging Committee.

Judges may abstain from voting for whatever reason.

If Exhibitors feel the need to discuss any decision by the Judges they should speak to the Judging Committee Chairman; other Judges are not at liberty to divulge any discussions or decisions made during judging.

EOCCE 2018 Judging Rules

All Judges and Stewards should carefully read the Judge's Handbook and bring their copy to the Show.

Exhibitors and anyone not involved in the judging process are required to leave the Hall while judging takes place.

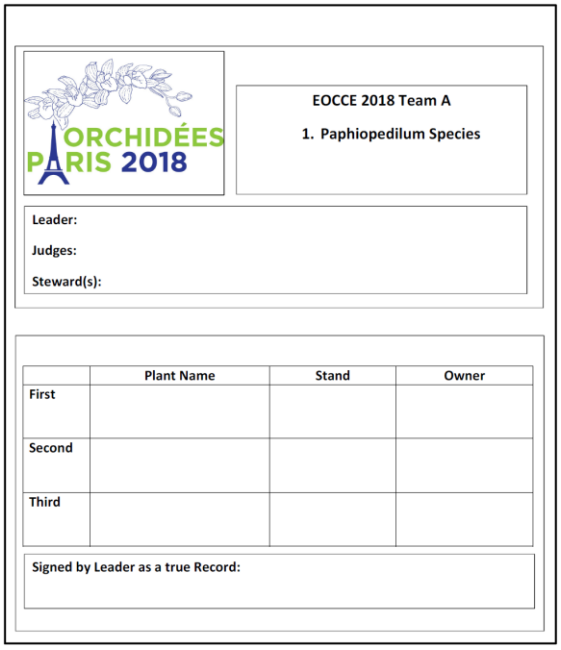

Figure 1. Example of form for Ribbon judging

All orchids on display in the Show are eligible to be judged in their appropriate classes, plants on sales tables cannot be judged.

Judges or Stewards may not change Teams without permission of the Judging Committee.

Where for any reason the votes for and against a particular award are even, the Team Leader shall have a casting vote.

Plants or cut flower spikes which show active disease or infestation will not be eligible for award, the Exhibitor may be asked to remove it from the hall. This decision is entirely that of the Judging Committee and its advisors.

Plants which show clear evidence of being recently wild-collected are excluded by the Show Rules and are not eligible for award. All plants on display should comply with current CITES and Plant Health regulations.

Where inadequate or incorrect labelling of an orchid prevents knowledge of the orchid's breeding or provenance, the orchid may be excluded from judging.

It is important to note that the use of flowering non-orchidaceous plants is not encouraged in displays.

The Organizers will exercise all appropriate caution in securing the exhibition and conference facilities at all times; however, Exhibitors and other conference participantsare solely responsible for the safety and security of their possessions before, during and after the show. The Organisers will not be liable for any loss or damage however caused. Exhibitors and Judges are strongly advised not to leave their goods unattended. No one may hold the Organizers, Sponsors or supporters, the Show or its Committees and workers (volunteers included) responsible for loss of, or to, plants, flowers, or any other item brought to the show by a participant, visitor or any person.

Plants put in displays after judging has started will not be eligible for judging.

All plants must remain on the display until the end of the show; otherwise any ribbon or trophy may be withheld.

Deliberately disbudded and overly manipulated plants will be disqualified.

In the event of any dispute or failure to reach a conclusion on any point, the Chairman of Judging will, together with the Judging Committee, ensure that a decision is made. Their decision will be final.

Judges and Stewards must wear their name label at all times during judging.

Smoking is strictly prohibited in the Hall.

All discussions during judging are confidential.

All official deliberations should be in English or be translated into English.

Exhibitors may not rename plants already possessing a valid recognised name and any wilful disregard of this will be taken into account in judgement.

Taxonomic rules to be used for the purposes of this event are those laid down on the RHS International Orchid Register and the World Check List of Selected Plant Families.

Judges who register after the deadline date of 1st March 2018 may be assigned to a Judging Team at the discretion of the Judging Committee. Judges who register less than a week before the show may not be able to be accommodated. Judges who present themselves unannounced can only be assigned to a Team by the Chairman of the Judging Committee and the Team Leader and will have no voting rights.

Definitions

An **Accredited Judge** is one recognised and certified as a Judge by a National Judging organization. This is subject to assessment by the Judging Committee.

A **Class** is a group of genera listed together (see schedules) to be judged in one group against one another.

A **Disbudded** plant is one where one or more buds have been removed from an inflorescence to increase size of the remaining blooms or to improve its configuration.

The **EOCCE 2018 Judging Committee** consists of a majority of the Members listed above and it's Chairman.

The **Final Judging Panel** is the group of Team Leaders to award Premier Trophies.

A **Steward** (Clerk) is attached to every Team to find plants, keep records, put out ribbons and assist with other duties. Normally this will be a Trainee Judge.

The **Team Leader** (Team Captain) is the person listed in the documentation to lead a particular Judging Team.

A **Trainee Judge** is one recognised as a Trainee or Probationary Judge by a National Judging Organization. A Trainee Judge does not have voting rights.

Medals and trophies

Trophies are provided in Tables 3 and 4. Winners are classified into several categories (Tables 5-7).

Master Class - EOCCE Judging Symposium

On the day after the judging, all the judges were invited to a symposium. One of its objectives was to compare the different judging schemes. It included practical exercises and presentations about the different systems

After a short introduction on why such a symposium was organized, Vinciane Dumont asked some participants to explain about the judging systems each European country used. David Lafarge, explained for France, Clare Hermans (RHS) and Chris Barker (BOC) for Great Britain, Carsten Hammer for Germany, Viviane Parrat for Switzerland, Guido Diana for Italy, Jan H. Larsen for Denmark.

After these short presentations, whatever system is used, the best is selected by most judging teams. The systems are working following parallel lines and get to the same result.

A practical judging session for all judges and trainees took place after this and it was very successful. Each participant could compare his results with known international judges who then gave reasons for their choice. Most of the participants agreed on the « bad » plants with defects, to choose the best was more complicated. This explains why a long practical training is necessary before becomming a judge. There followed many questions and an open and frank discussion making the session a great success.

In conclusion the participants asked for more symposiums in the same style

Table 3. Plant trophies.

No	Title	Notes
30	**Best Cypripedioideae (slipper orchid)**	**Trophy** best of classes **1-4**
31	**Best Cyrtopodiinae**	**Trophy** best of classes **5-6**
32	**Best Lycastinae – Maxillariinae - Zygopetalinae**	**Trophy** best of classes **7-8**
33	**Best Oncidiinae**	**Trophy** best of classes **9-10**
34	**Best Coelogyninae**	**Trophy** best of classes **11-12**
35	**Best Laeliinae**	**Trophy** best of classes **13-14**
36	**Best Pleurothallidinae**	**Trophy** best of classes **15-16**
37	**Best Dendrobiinae**	**Trophy** best of classes **17-18**
38	**Best Vandeae (not including *Phalaenopsis*)**	**Trophy** best of classes **20-23**
39	**Best *Phalaenopsis***	**Trophy** best of classes **24-26**
40	**Best Any Other**	**Trophy** best of classes **19, 27, 28, 29**

Table 4. Premier trophies.

No	Title	Notes
41	**Best species**	**Premier Trophy**
42	**Best hybrid**	**Premier Trophy**
43	**Grand Champion Plant in Show**	**Premier Trophy** best of classes **41 &-42**

Table 5. EOC2018 Premier trophy winners – Plants.

Class	Title	Plant	Owner
41	Best species	***Oncidium cristatum***	**Writhlington School**
42	Best hybrid	***Dendrobium* x *delicatum***	**La Canopée**
43	Grand Champion Plant in Show	***Oncidium cristatum***	**Writhlington School**

Table 6. EOC2018 Trophy winners – Plants.

Class	Title	Plant	Owner
30	Best Cypripedioideae (Slipper Orchid)	***Paphiopedilum* Wössner Vietnam Star**	**L'Amazone**
31	Best Cyrtopodiinae	***Cymbidium madidum***	**La Cour des Orchidées**
32	Best Lycastinae - Maxillariinae - Zygopetalinae	***Lycaste* Denley**	**La Canopée**
33	Best Oncidiinae	***Oncidium cristatum***	**Writhlington School**
34	Best Coelogyninae	***Coelogyne cristata***	**Writhlington School**
35	Best Laeliinae	***Prosthechea* Scriptum* (Prismatocarpax*Cochleata)**	**L'Amazone**
36	Best Pleurothallidinae	***Masdevallia uniflora***	**Akerne Orchids K.Bruyninck**
37	Best Dendrobiinae	***Dendrobium* x *delicatum***	**La Canopée**
38	Best Vandeae (not including *Phalaenopsis*)	***Vanda ampullacea* 'Dario'**	**EOC - FIO**
39	Best *Phalaenopsis*	***Phalaenopsis mannii***	**Writhlington School**
40	Best Any Other	***Cypripedium formosanum***	**EOC – Michael Tibbs**

Table 7. EOC2018 Ribbon winners.

Class	Title	Plant	Owner
1.1	*Paphiopedilum* species. First	*Paphiopedilum rothschildianum*	Popow Orchids
1.2	*Paphiopedilum* species. Second	*Paphiopedilum haynaldianum*	Asendorfer
1.3	*Paphiopedilum* species. Third	*Paphiopedilum delenatii* f. *vinicolor*	Vacherot Lecoufle
2.1	*Paphiopedilum* Hybrid. First	*Paphiopedilum* Wössner Vietnam Star	L'Amazone – Gérard Schmidt
2.2	*Paphiopedilum* Hybrid. Second	*Paphiopedilum* White Lady	Asendorfer

Class	Title	Plant	Owner
2.3	*Paphiopedilum* Hybrid . Third	*Paphiopedilum* Arthurianum	Jardin du Luxembourg - Sénat
3.1	*Phragmipedium* species. First	*Phragmipedium lindleyanum*	Jardin du Luxembourg - Sénat
3.2	*Phragmipedium* species. Second	*Phragmipedium caudatum*	LOF- L'Orchidée en France
3.3	*Phragmipedium* species. Third	*Phragmipedium richteri*	Asendorfer
4.1	*Phragmipedium* Hybrid. First	*Phragmipedium* Fritz Schomburg "Alberto"	EOC - FIO. Federazione Italiana
4.2	*Phragmipedium* Hybrid. Second	*Phragmipedium* Don Wimber	Vacherot Lecoufle
4.3	*Phragmipedium* Hybrid. Third	*Phragmipedium* Wössner Supergrande	Vacherot Lecoufle
5.1	Cyrtopodiinae species. First	*Cymbidium madidum*	La Cour des Orchidées
5.2	Cyrtopodiinae species. Second	*Cymbidium erythraeum*	Jardin du Luxembourg - Sénat
5.3	Cyrtopodiinae species. Third	*Cymbidium devonianum*	Writhlington School
6.1	Cyrtopodiinae hybrid. First	*Cymbidium* Gymer 'Cooksbridge'	Orchideen Garten Marei Karge
6.2	Cyrtopodiinae hybrid. Second	*Cymbidium* Cricket (*devonianum* x *madidum*)	La Cour des Orchidées
6.3	Cyrtopodiinae hybrid. Third	*Cymbidium* Hoosailum (*floribundum* x *sinense*)	La Cour des Orchidées
7.1	Lycastinae, Maxillariinae & Zygopetalinae species. First	*Lycaste skinneri*	Jardin du Luxembourg - Sénat
7.2	Lycastinae, Maxillariinae & Zygopetalinae species. Second	*Lycaste virginalis*	La Canopée
7.3	Lycastinae, Maxillariinae & Zygopetalinae species. Third	*Maxillaria lepidota*	Les Orchidées de Michel Vacherot
8.1	Lycastinae ,Maxillariinae & Zygopetalinae Hybrid. First	*Lycaste* Denley	La Canopée
8.2	Lycastinae, Maxillariinae & Zygopetalinae Hybrid. Second	*Angulocaste* Olympus 'Rex'	L'Amazone
8.3	Lycastinae & Maxillariinae Hybrid. Third	*Zygopabstia* Elaine Oliver x *Zygopetalum* Blue Blood	EOC
9.1	Oncidiinae species. First	*Oncidium cristatum*	Writhlington School
9.2	Oncidiinae species. Second	*Miltonia kayasimae*	L'Amazone
9.3	Oncidiinae species. Third	*Oncidium maculatum*	Vacherot Lecoufle
10.1	Oncidiinae Hybrid. First	*Oncidium* Avalon	Vacherot Lecoufle

Class	Title	Plant	Owner
10.2	Oncidiinae Hybrid. Second	*Oncidium* Widecombe Fair 'Burnham'	Vacherot Lecoufle
10.3	Oncidiinae Hybrid. Third	*Miltoniopsis* Robert Strauss 'White Flag'	EOC Michael Tibbs
11.1	Coelogyninae species. First	*Coelogyne cristata*	Writhlington School
11.2	Coelogyninae species. Second	*Coelogyne holochila*	Writhlington School
11.3	Coelogyninae species. Third	*Coelogyne cristata*	Hans Christiansen
12.1	Coelogyninae Hybrid. First	*Coelogyne* Orchideengarten Sabine	Orchideen Garten Marei Karge
12.2	Coelogyninae Hybrid. Second	*Coelogyne* Intermedia	EOC - FIO
12.3	Coelogyninae Hybrid. Third	*Coelogyne* Orchideengarten Clara	Orchideen Garten Marei Karge
13.1	Laeliinae species. First	*Epidendrum parkinsonianum*	Writhlington School
13.2	Laeliinae species. Second	*Epidendrum centropetalum*	Writhlington School
13.3	Laeliinae species. Third	*Epidendrum stamfordianum*	Les Orchidées de Michel Vacherot
14.1	Laeliinae Hybrid. First	*Prosthechea scriptum* x (*Psh. prismatocarpa* x *Psh. cochleata*)	L'Amazone – Gérard Schmidt
14.2	Laeliinae Hybrid. Second	*Cattleya* Thais de Valec	Vacherot Lecoufle
14.3	Laeliinae Hybrid. Third	Cattlianthe Chit Chat 'Tangerine'	Associazione Italiana di Orchidologia
15.1	Pleurothallidinae species First	*Masdevallia uniflora*	Akerne Orchids K.Bruyninck
15.2	Pleurothallidinae species. Second	*Porroglossum meridionale*	Writhlington School - Simon Pugh-Jones
15.3	Pleurothallidinae species. Third	*Masdevallia ignea*	LOF – Raoul Cere
16.1	Pleurothallidinae Hybrid. First	*Masdevallia* Pichincha	Akerne Orchids K.Bruyninck
16.2	Pleurothallidinae Hybrid. Second	----------	
16.3	Pleurothallidinae Hybrid. Third	----------	
17.1	Dendrobiinae species. First	*Dendrobium gracilicaule*	La Canopée
17.2	Dendrobiinae species. Second	*Dendrobium mohlianum*	Writhlington School
17.3	Dendrobiinae species. Third	*Dendrobium tetragonum*	EOC - FIO
18.1	Dendrobiinae Hybrid. First	*Dendrobium* x *delicatum*	La Canopée

Class	Title	Plant	Owner
18.2	Dendrobiinae Hybrid. Second	*Dendrobium* Mtn's Butterfly Kisses (*cuthbertsonii* x *glomeratum*)	Hans Christiansen
18.3	Dendrobiinae Hybrid. Third	*Dendrobium* (*alexandrae* x *rhodostictum*)	Asendorfer
19.1	Bulbophyllinae species or hybrid. First	*Bulbophyllum falcatum*	La Canopée
19.2	Bulbophyllinae species or hybrid. Second	*Bulbophyllum falcatum*	Röllke
19.3	Bulbophyllinae species or hybrid. Third	*Bulbophyllum graveolens* 'Big Brother'	Röllke
20.1	Aeridinae species (not including Phalaenopsis). First	*Vanda ampullacea* "Dario"	EOC - FIO
20.2	Aeridinae species (not including Phalaenopsis). Second	*Vanda lamellata* var. *remediosae*	EOC - FIO
20.3	Aeridinae species (not including Phalaenopsis). Third	*Papilonanthe vandarum*	Jardin du Luxembourg - Sénat
21.1	Aeridinae Hybrid (not including Phalaenopsis). First	*Vanda* Sunanda Magic Black	Orchideen Garten Marei Karge
21.2	Aeridinae Hybrid (not including Phalaenopsis). Second	----------	
21.3	Aeridinae Hybrid (not including Phalaenopsis). Third	----------	
22.1	Aerangidinae & Angraecinae species. First	*Angraecum sesquipedale*	Vacherot Lecoufle
22.2	Aerangidinae & Angraecinae species. Second	*Jumellea arachnantha*	Marei Karge
22.3	Aerangidinae & Angraecinae species. Third	*Angraecum sesquipedale*	La Canopée
23.1	Aerangidinae & Angraecinae Hybrid. First	*Aerangis* Zipper	Vacherot Lecoufle
23.2	Aerangidinae & Angraecinae Hybrid. Second	----------	
23.3	Aerangidinae & Angraecinae Hybrid. Third	----------	

Class	Title	Plant	Owner
24.1	*Phalaenopsis* species. First	*Phalaenopsis mannii*	Writhlington School
24.2	*Phalaenopsis* species. Second	*Phalaenopsis schilleriana*	L'Amazone - Gérard Schmidt
24.3	*Phalaenopsis* species. Third	*Phalaenopsis lobbii*	Ecuagenera
25.1	*Phalaenopsis* Hybrid – Plain. First	*Phalaenopsis* Sexy Venus 'Chaplin'	Joseph Wu
25.2	*Phalaenopsis* Hybrid - Plain. Second	*Phalaenopsis* Demoiselle de Rochefort	Vacherot Lecoufle
25.3	*Phalaenopsis* Hybrid - Plain. Third	*Phalaenopsis* Crème Chantilly	Vacherot Lecoufle
26.1	*Phalaenopsis* Hybrid – Patterned. First	*Phalaenopsis* Sexy Venus 'Nancy'	Joseph Wu
26.2	*Phalaenopsis* Hybrid – Patterned. Second	*Phalaenopsis* Little One (*hygrochila* x *japonica*)	LOF
26.3	*Phalaenopsis* Hybrid – Patterned. Third	*Phalaenopsis* Yaphon Goodboy x *Phalaenopsis gigantea*	Orchid4U
27.1	Any other Terrestrial species or Hybrid. First	*Cypripedium formosanum*	EOC – Michael Tibbs
27.2	Any other Terrestrial species or Hybrid. Second	*Pterostylis curta*	Akerne Orchids K. Bruyninck
27.3	Any other Terrestrial species or Hybrid. Third	*Dienia ophrydis*	Röllke
28.1	Any other species not included above. First	*Dendrochilum wenzelii*	EOC – Dansk Orchidee Club
28.2	Any other species not included above. Second	*Chysis bractesens*	Les Orchidées de Michel Vacherot
28.3	Any other species not included above. Third	*Oeceoclades saundersiana*	L'Orchidium
29.1	Any other Hybrid not included above. First	*Calanthe* (*sieboldii* x *discolor*)	L'Amazone – Gérard Schmidt
29.2	Any other Hybrid not included above. Second	*Zygoneria* Dynamax x *Zygopetalum* Titanic	EOC Michael Tibbs
29.3	Any other Hybrid not included above. Third	*Calanthe* Kozu	EOC Michael Tibbs

Lecture programe

SATURDAY, MARCH 24

9:00 ..... *REGISTRATION AND OPENING CEREMONY*

9:30 Session Conservation and restoration in a changing world Chair: Jana Jersáková

Tiiu Kull ..... Conservation status, reproduction biology and restoration need of the European emblematic orchid

Pavel Kindlmann ..... Species distribution models and their application in orchid biodiversity research

Sonja Hurskainen ..... Tree removal as a management strategy for the lady's slipper orchid

COFFEE BREAK

11:10 Ekaterina Zheleznaia .... The mosaic cycles of ecosystems and population strategies of terrestrial orchids

Mike F. Fay ..... The IUCN Orchid Specialist Group in the new Quadrennium

Philippe Feldmann ..... Improvement of evaluation of the extinction risks of the French wild orchids using citizen science's shared data

Jean-Michel Hervouet.... The ongoing story of Ambodiriana forest in Madagascar, a representative case study of in situ conservation

Daniel Tyteca ..... A synthesis of recent trends in *Platanthera* (Orchidaceae) systematics in Western Europe

13:00 ..... *LUNCH*

14:00 Poster session (odd numbers)

15:00 Session Ecology of mutualism - Chair: Richard Bateman

Marc-André Selosse ..... Mycoheterotrophy and mixotrophy in orchids: an update

Mariangela Girlanda ..... Where do orchid mycorrhizal fungi come from?

Jana Jersáková ..... Untangling factors underlying distribution of forest orchids

Julita Minasiewicz ..... Low within-species specificity for fungal partner in the rare mycoheterotrophic orchid *Epipogium aphyllum* Sw.

COFFEE BREAK

17:00 Félix Lallemand ..... The evolution of mycoheterotrophy in Neottieae

Etienne Delannoy..... Integrated omics analysis of natural albino orchids to decipher the physiological adaptations of mixotrophs

ShiCheng Shao..... An effective and practical tool for orchid reintroduction and conservation

GALA DINNER
AWARD CEREMONY

SUNDAY, MARCH 25

9:15 Session Ecology of mutualism (continued) - Chair: Marc-André Selosse

Giovanni Scopece . . . . . Pollination efficiency and the evolution of specialized deceptive pollination systems in orchids

Monika Lipińska . . . . . Pollination strategies in Neotropical genus *Maxillaria sensu lato* – chemical and micromorphological analysi of floral attractants and their potential biological implications

Bertrand Schatz . . . . . Ecological and environmental factors affecting of fruit set among Euro-Mediterranean orchids

COFFEE BREAK

10:45 Session Orchids in the era of genomics - Chair: Marc-André Selosse

Barbara Gravendeel . . . . . The orchid genomic toolkit

Mark W. Chase . . . . . Confronting the genomics revolution head-on: what we can learn about what problems are bothering orchid species

Richard Bateman . . . . . Is genetic technology approaching the limit of its ability to help us understand the systematic biology of orchids?

Hans Jacquemyn . . . . . Genetic divergence and ecotype formation in *Epipactis*

Silvia Perotto . . . . . Multi-omics approaches provide insights into fungal-plant interactions in the model system *Serapias vomeracea - Tulasnella calospora*

Kenneth Cameron . . . . . Genome size and phylogenetics of Vanilloideae (Orchidaceae) inferred from NextGen anchored phylogenomics

13:00 . . . . . *LUNCH*

14:00 Poster session (even numbers)

15:00 . . . . . Oral presentation for awarded posters

15:30 Session Biotechnology and breeding - Chair: Daniel Prat

Hong-Hwa Chen . . . . . Orchid biotechnology and breeding

COFFEE BREAK

16:30 Wen-Huei Chen . . . . . Achievements of *Phalaenopsis* Orchid Breeding in Taiwan

Michel Grisoni . . . . . *Vanilla*: a challenging genus with regards to the development of genomic resources

Juan Chen . . . . . Transcriptome and proteome revealed the symbiotic germination mechanism of *Dendrobium officinale* (Orchidaceae) inoculated with *Tulasnella* sp.

17:30 . . . . . *CLOSING CEREMONY*

Author index

Ochid species index

(investigated and cited species; taxonomy according to autors)

Publications from the French Orchid Society (SFO)

Journals:
L'Orchidophile
more than 200 issues published since 1970
4 issues a year

Cahiers de la Société Française d'Orchidophilie
N° 1 (1993) : *Synopsis des orchidées européennes*, by Pierre Quentin
N° 2 (1995) : *Synopsis des orchidées européennes*, deuxième édition, by Pierre Quentin
N° 3 (1996) : *Actes du 13ème colloque de la SFO*, Grenoble, 29 juin – 2 juillet 1995
N° 4 (1999) : *Compte-rendu des premières journées rencontres orchidophiles Rhône-Alpes*, Lyon, 30 mai-1er juin 1998
N° 5 (1999) : *Les hybrides des genres* Nigritella *et/ou* Pseudorchis, by O. Gerbaud et W. Schmid (coédition SFO-AHO)
N° 6 (2000) : *Actes du 14e colloque de la SFO*, Paris, 20-21 novembre 1999
N° 7 (2010) : *Actes du 15e colloque sur les orchidées de la Société Française d'Orchidophilie*, Montpellier, 30 mai - 1er juin 2010
N° 8 (2014) : *Actes du 16e colloque sur les orchidées de la Société Française d'Orchidophilie, Quel avenir pour les orchidées dans leur milieu ?* Blois, 1-2 mars 2014
N° 9 (2018) : *18th European Orchid Council Conference and Exhibition Proceedings*, Paris 2-25 March 2018

Orchid mapping
18 issues published as supplements to *L'Orchidophile* focussed on orchid distribution within a single French department
More than 15 other issues or booksat department or regional level, sometime in co-edition with other partners

Books
Various books on temperate and tropical orchids, including :
Les orchidées de France, Belgique et Luxembourg. 2005. (M. Bournérias et D. Prat, coordinateurs)
Atlas des orchidées de France. 2010. (F. Dusak et D. Prat, coordinateurs)
Sabots de Vénus, orchidées fascinantes. 2013. (Collectif SFO, supplément à l'Orchidophile)

La Société Française d'Orchidophilie, fondée en 1969, a pour objectifs majeurs :
- d'étudier la répartition et l'écologie des Orchidées en France et dans d'autres pays ;
- de protéger les espèces sauvages les plus menacées ;
- de favoriser la culture des espèces horticoles ;
- d'encourager les études sur la biologie des orchidées.

Ces objectifs sont atteints grâce :
- à des réunions et colloques ;
- à des voyages d'étude ;
- au réseau de cartographes ;
- aux activités régionales menées dans les associations locales affiliées ;
- aux publications (bulletin, cartographies, ouvrages).

The "Société Française d'Orchidophilie" (French Orchid Society), formed in 1969, aims the main following activities:
- studying orchid distribution and ecology in France and everywhere else;
- protecting most endangered wild species;
- promoting cultivation of horticultural species;
- encouraging studies on orchid biology.

These goals are reached through:
- meetings and symposiums;
- field trips;
- network of cartographers;
- local activities of regional affiliated associations;
- publications (bulletin, cartographies, books).