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## **Evolutionary diversification and historical biogeography of orchidaceae in Central America with emphasis on Costa Rica and Panama**

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# **General discussion and conclusions**



# Chapter 10

## General discussion and conclusions

In this chapter, I discuss further steps needed to compliment the findings of this thesis and the work that must be continued in a general perspective to understand orchid species radiations in the Neotropics from three main topics: (i) taxonomy, systematics and evolution of floral traits, (ii) pollination biology and animal interactions and (iii) biogeography and evolutionary history.

### 10.1 Taxonomy, systematics and evolution of floral traits

The current angiosperm diversification in the Neotropics needs to be approached from an integrative perspective involving different sources of information. One of the most basic sources is alpha-taxonomy, primarily based on detailed morphological documentation of plants coupled with accurate locality data. This stage is the starting point to design and infer solid, densely sampled phylogenies which are appropriate to test further evolutionary hypotheses on species diversifications. However, an accurate phylogenetic sampling of *Lepanthes* is still a challenge because the number of species is overwhelming and new species continue to be discovered throughout the Neotropical region, thus producing a subestimation of the sampled lineages within the phylogenies. Therefore, alpha-taxonomic studies and botanical exploration should be made a priority to improve phylogenetic sampling as well as knowledge on species distributions. As we have shown in chapter 2 and 3, integrative taxonomy is also key in addressing species complexes difficult to separate morphologically. In this thesis, I described four new species of *Lepanthes*, two recognized primarily on morphology and two supported by morphology and a well-resolved phylogeny based on hundreds of innovative molecular markers. This was just a small contribution to the current taxonomic impediment, and I hope the discovery of additional new species will be sped up by exciting new methodologies such as for instance orchid pictures posted on social media or websites like iNaturalist (<https://www.inaturalist.org>) by orchid enthusiasts, naturalists or tourists (Kusuma-Wati et al., 2018).

Another challenge in understanding hyperdiverse orchid lineages such as *Lepanthes* is the explosive diversifications that resulted in poorly supported nodes and morphological convergence across clades. In these cases, inferences based on few molecular markers such as the traditional nrITS and matK regions are insufficient to provide clear species relationships in complexes of recent and rapid diversifications. Here, I demonstrated that high-throughput sequencing techniques such as anchored hybrid enrichment coupled with coalescence-based methods is a powerful tool to solve complicated phylogenetic relationships in lineages derived from recent, rapid diversifications. Furthermore, phylogenomic datasets provide additional information on biological phenomena such as incomplete lineage sorting, hybridization or polyploidy that might cause discordance among individual gene trees. I found that only with a large number of innovative phylogenetic markers generated from three different genomes, the phylogeny of the *L. horrida* complex could be fully resolved and this enabled us to separate traits evolving in parallel or convergently across

these orchid lineages, such as flower color and size, from evolutionary informative diagnostic traits such as the shape and orientation of the lobes of the petals and lip. In conclusion, I recommend that more phylogenomic datasets should be generated for resolving more challenging groups within the genus *Lepanthes* and the Pleurothallidinae in general. Likewise, targeting the most informative phylogenetic markers obtained with phylogenomic datasets would be an adequate strategy to increase the sampling in hyperdiverse groups because analyzing large datasets of hundreds of species and markers might be computationally arduous. Most previous phylogenetic sampling in the Pleurothallidinae subtribe relied on two regions (nrITS and matK), therefore, the development of innovative markers such as those generated during this PhD thesis will be very useful for future phylogenetic studies of the orchid family. The advent of exciting new genomic tools such as target capture-based methods and transcriptomics in combination with custom made bioinformatics pipelines (Gravendeel et al., 2018) will definitely speed up this process. So far we conclude that alpha-taxonomy and the use of new techniques such as high-throughput sequencing are useful tools to clarify inter-specific relationships. However, another of the challenges of this thesis was to clarify the puzzling intergeneric relationships of the *Lepanthes* clade. Again, insufficient taxonomic sampling of clades precluded previous attempts to clarify generic relationships. However, the lack of a congruent system in assessing suitable morphological traits is still confusing the generic delimitations in the Pleurothallidinae. Generic delimitations solely based on morphological characters are daunting because of overwhelming homoplasy of the characters traditionally used for circumscriptions. I provided evidence for recognizing 14 well supported genera as members of the clade based on a combination of molecular phylogenetics and a solid morphological assessment identifying both synapomorphies and homoplastic characters. Future research should focus on sampling additional members of *Trichosalpinx* subgenus *Xenia*, which are extremely rare but need to be phylogenetically evaluated in order to obtain a complete evolutionary scenario for the *Lepanthes* clade. Based on morphology, we suspect that some members might be related to *Lepanthopsis* and allies but this hypothesis needs further evaluation. In addition, it is desirable to increase sampling in other groups such as *Lepanthopsis* (mainly the Antillean species) and *Tubella* because of floral similarities. Our phylogenetic framework and methodological approach enabled the discovery of useful traits for generic classifications, and paves the way for more comprehensive assessments on generic delimitations of similar recalcitrant lineages based on DNA sequences and morphological characters to further improve the systematics of the Pleurothallidinae. In conclusion, having a well resolved phylogeny and a fine delimitation of the clades is the starting point to explain the morphological evolution and the role of other biotic and abiotic factors in the diversification of the *Lepanthes* clade. This group challenged systematists and taxonomists for centuries due to the floral homoplasy untangled here, which is possibly resulting from similar pollination systems.

## 10.2 Pollination biology and animal interactions

The role of pollinators as drivers of species richness and morphological diversity is largely unknown because knowledge of pollination systems in Pleurothallidinae is still scarce. Pleurothallidinae are a megadiverse Neotropical orchid subtribe comprising > 5200 species, most of which are probably pollinated by Diptera. The *Lepanthes* clade accounts for about 25% of

the species of the subtribe but only one pollination system was described so far for the genus *Lepanthes*, with documented observations on just three species. I disclosed the pollination system of *Trichosalpinx*, which uses a completely different strategy compared to the *Lepanthes* pseudocopulatory system by male fungus gnats. The exclusive presence of female biting midges searching for proteins on the lip surface of *Trichosalpinx* and their well-developed mandibles and poorly developed laciniae indicate that they mainly feed on invertebrate hosts from which they draw haemolymph. Therefore, *Trichosalpinx* spp. might exclusively attract female midges by exploiting their protein collection instinct for egg production. The similar floral structures of other kleptomyophilous angiosperms compared to *Trichosalpinx* and the kleptoparasitic habits of *Forcipomyia* (Euprojoannisia) suggest that kleptomyiophily may have evolved in *Trichosalpinx*. This hypothesis derived from our study should be further tested by investigating the natural history of the *Forcipomyia* sp., discovery of the males, their feeding and breeding sites, diets and prey. Dietary analysis, bioassays and behavioural studies of both *Forcipomyia* sp. and their insect prey and GC/MS analyses of their pheromones and cuticular scents and the floral fragrance of other *Trichosalpinx* spp. are additional evidence needed. Most of the species of *Trichosalpinx* show similar floral traits, therefore, more observations in other species are necessary to confirm the pollination strategy revealed here. Likewise, the similarities among *Trichosalpinx* and the closely related *Anathallis* and *Lankesteriana* suggest that they also have similar pollination mechanisms. In addition to macromorphological similarities of the flowers of *Lankesteriana* and *Trichosalpinx* and some *Anathallis*, the species of these genera share micromorphological and histological characters that support a hypothesis of pollination by biting midges and thus parallelism. One of the most important shared characters is the secretion of proteins in the papillae of the lip and the striated cuticle of their epidermis. Species of *Trichosalpinx* employ this strategy to attract females of *Forcipomyia* for pollination and this might occur in *Lankesteriana* and some *Anathallis* as well. In the phylogenetic context, at least two families of Diptera are involved in the pollination of species in the *Lepanthes* clade: Sciaridae males in *Lepanthes* and Ceratopogonidae females in *Trichosalpinx*. However, the pollination mechanisms of the remaining 12 genera of the *Lepanthes* clade should also be investigated in order to obtain a complete picture of the evolution of pollination syndromes and floral traits related to them. These systems should be disclosed not only by describing the pollination system with innovative tools such as camera traps and Automatic Image Detection by machine learning but also by linking behavior and natural history of the pollinators to the strategy of attraction by the flowers. Consequently, other members of the group are likely pollinated by Diptera and in other groups such as *Tubella*, *Lepanthopsis* and *Zootrophion* the pollination systems are probably different from those already known. Apart from the pollination system, in *Anathallis*, *Fronitaria*, *Lankesteriana*, *Tubella*, *Trichosalpinx* s.l. and *Zootrophion*, the pollinarium is deposited on the thorax of the pollinator since the columns are long and arcuate with an incumbent anther and a pollinarium with sticky caudicles. In contrast, in *Lepanthopsis* the pollinarium is likely not deposited on the thorax of the pollinator since the column is short and bilobed and the flower therefore does not allow for an entrance and exit as described for the genera mentioned above. Therefore, *Lepanthopsis* might employ a similar pollination strategy as *Platystele*, *Stelis* or *Pleurothallis*. Despite the availability of a described pollination system for three species of *Lepanthes*, more observations on other species are needed in order to fully understand the role of pollinators on the diversification and evolution of floral traits. During my

PhD project, I obtained preliminary data on pollination of *Lepanthes jugum* Luer and these data suggest that this species uses the pollinator's body in a different way as *L. glicensteinii* and *L. yubarta* to adhere its pollinarium to. In addition, the hypothesis of imitation of insect pheromones by *Lepanthes* flowers to attract male fungus gnats needs to be further tested. Finally, X-ray micro-computed tomography of orchid pollinators preserved in amber fossils might improve our knowledge on the timing of the evolution of this type of deceptive pollination as the fossil record of Ceratopogonidae is one of the best conserved among Diptera (Borkent and Spinelli, 2007).

### 10.3 Biogeography and evolutionary history

Based on two extensively sampled orchid phylogenies, combined with statistically robust diversification models, our results reveal that orchid diversification has closely tracked the Andean-Central American orogeny. Together with studies in other mega-diverse regions (Bruyn et al., 2014; Verboom et al., 2009), our results show that rapid recent speciation has moulded this area of exceptional species richness. In addition, our results highlight the crucial role of Amazonian lowlands, as well as the Antillean and Central American regions, as biotic sources for Andean, Northern Central America and Antillean biodiversity, providing cool pre-adapted lineages that dispersed into the Andes and further diversified *in situ*. The rise of the Andes had little effect on restricting orchid biotic dispersal across the Neotropics, suggesting that mountains are semi-permeable barriers to lowland organisms, whose dispersal ability is more probably related to intrinsic traits (e.g. seed size, dispersal mechanism, mutualisms). Although both abiotic and biotic processes are clearly responsible for the exceptional species richness of the world's premier biodiversity hotspot (Antonelli and Sanmartín, 2011; Hughes et al., 2013; Eiserhardt et al., 2017), our results suggest that geological processes played a central and direct role in the diversification process. Finally, as the highest species richness in Cymbidieae is concentrated in the lowlands and the Pleurothallidinae peak is at mid-elevation, our study shows that Andean uplift dramatically affected the evolutionary assembly of both lowland and mid-elevation Andean forests, as originally hypothesized by (Gentry, 1982). The genus *Lepanthes* likely originated during the Miocene in the Central Andes and reached Southern Central America from the Andean region twice. The extant lineages from Northern Central America and the West Indies were likely derived from Southern Central American MRCA's and not from Guyana or Amazonia suggesting that the isthmus of Panama served as a land bridge for lineages derived from Andean ancestors.

Future taxonomical and biogeographical research should focus on obtaining more accurate species distributions. For example, knowledge on orchid distributions and flowering periods can be improved by extracting image metadata from pictures (GPS location coordinates, elevation and time stamps) using command-line applications like Exiftool (<https://www.sno.phy.queensu.ca/~phil/exiftool/>). In addition, increasing taxon sampling and include multi-locus approaches to further test the influence of geographical barriers on current diversity patterns. Furthermore, other Neotropical areas such as northern Central America and the Antilles require more biogeographical research to complement our findings. The role of extant important biomes (most notably montane forests) in the diversification of the most diverse orchid groups within the Pleurothallidinae will then likely become much clearer. Such a result will have main conservation applications because a major group of orchids and associated pollinators and hosts are vulnerable to global warming, especially in cold, high-elevation areas, where they are most diverse.