Molecular Phylogenetics, Evolution of Sexual Systems and Historical Biogeography of Darwin's Favourite Orchids (Catasetinae) and Swan Orchids (*Cycnoches* Lindl.)

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Oscar Alejandro Pérez-Escobar
München, April 2016

"Home is behind, the world ahead,
and there are many paths to tread
through shadows, to the edge of night,
until the stars are all alight"
-J.R.R. Tolkien, The Lord of the Rings

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April, 2016

Cover: *Cycnoches pentadactylon* Lindl. illustrated by the author.

EIDESSTATTLICHE VERSICHERUNG UND ERKLÄRUNG

Diese Dissertation wurde im Sinne von §12 der Promotionsordnung von Prof. Dr. Marc Gottschling betreut. Ich erkläre hiermit, dass die Dissertation nicht einer anderen Prüfungskommission vorgelegt worden ist und dass ich mich nicht anderweitig einer Doktorprüfung ohne Erfolg unterzogen habe.

Ich versichere hiermit an Eides statt, dass die vorgelegte Dissertation von mir selbstständig und ohne unerlaubte Hilfe angefertigt wurde.

Oscar Alejandro Pérez-Escobar, 13th April 2016

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LIST OF PUBLICATIONS

Peer-reviewed journal articles

PÉREZ-ESCOBAR, O.; KOLANOWSKA, M. AND E. PARRA. 2013. *Lepanthes elizabethae* (Pleurothallidinae, Orchidaceae), a new species from Colombia. *Phytotaxa* 79: 58-62

PÉREZ-ESCOBAR, O. AND M.A. BLANCO. 2014. Rediscovery of *Malaxis nana* (Orchidaceae: Malaxideae) in Costa Rica. *Lankesteriana* 14: 109-114.

PÉREZ-ESCOBAR, O.; BALBUENA, J.A. AND M. GOTTSCHLING. 2016. Rumbling orchids: how to assess divergent evolution between chloroplast endosymbionts and the nuclear host. *Systematic Biology* 65: 51-65

PÉREZ-ESCOBAR, O.; GOTTSCHLING, M.; WHITTEN, M.W.; SALAZAR, G. AND G. GERLACH. 2016. Sex and the Catasetinae (Darwin's favourite orchids). *Molecular Phylogenetics and Evolution* 97: 1-10

PÉREZ-ESCOBAR, O.; GOTTSCHLING, M. AND G. GERLACH. *In revision*. Historical biogeography of *Cycnoches* (Catasetinae): the improbable journeys of Sawn Orchids across the Andes. *Journal of Biogeography.*

Monograph

HÁGSATER, E.; SANTIAGO-AYALA, E.; **PÉREZ-ESCOBAR, O.**; SALDAÑA-SÁNCHEZ, L.; COLLANTES, B.; VALDIVIESO, P.; CHOCCE-PEÑA, M.; SÁNCHEZ, E.; KARREMANS, A.; GONZALEZ, R.; MENEGUZZO, T.; KOLANOWSKA, M.; TARAZONA, M.; ÁLVAREZ, L.; DALSTRÖM, S.; DODSON, C.; FERNÁNDEZ, M.; GARCÍA, D.; MEDINA, H.; MORMONTOY, R.; NAURAY, W.; RINCÓN-USECHE, C.; RUÍZ, S.; SERGUERA, M.; SMITH, C.; VILLAFUERTE, M.; VEGA, N. AND F. WERNER. 2015. Icones Orchidacearum Fascicle 14. The genus *Epidendrum*, Part 10: Species new and old in *Epidendrum*. Herbarium AMO. Instituto Chinoin, A.C. Mexico City, 209 p.

DECLARATION OF CONTRIBUTION AS CO-AUTHOR

In this thesis, I present the results from my doctoral research, carried out in Munich (Germany) from April 2012 to July 2016, under the guidance of Prof. Dr. Marc Gottschling. My thesis resulted in six manuscripts presented in Chapters 2 to 7, of which five have been published (Chapters 2 to 6), and one is *in revisio* (Chapter 7). I also gave conference talks and poster presentations listed below. I generated all data and conducted all analyses myself, except for the *in-silico* simulations and pipeline scripting (part of Chapter 5), which was done in collaboration with Dr. Juan Antonio Balbuena (University of Valencia, Spain), the morphological descriptions of new *Epidendrum* species (Chapter 4), which was done with the help of Eric Hágsater, Elizabeth Santiago-Ayala and Luis Sánchez Saldaña (Herbarium AMO, Mexico), and observations on orchid phenology and reproduction (Chapter 6), which were done in collaboration with Dr. Gerardo Salazar (Universidad Nacional Autonoma de Mexico, Mexico). Writing and discussion involved collaboration with Prof. Dr. Marc Gottschling, Dr. Günter Gerlach and Dr. Mario Blanco. Detailed contributions to publications are provided as follows:

Chapter II

Pérez-Escobar, O.; Kolanowska, M. and E. Parra (2013) *Phytotaxa* 79: 58-62

Own contribution: Field work (80%); morphological analysis (including plates and illustration: 100%); manuscript preparation (80%).

Chapter III

Pérez-Escobar, O. and M.A. Blanco (2014) *Lankesteriana* 14: 109-114

Own contribution: Field work (100%); morphological analysis (including plates and illustration: 80%); manuscript preparation (60%).

Chapter IV

Hágsater, E.; Santiago-Ayala, E.; **Pérez-Escobar, O.**; Saldaña-Sánchez, L.; Collantes, B.; Valdivieso, P.; Chocce-Peña, M.; Sánchez, E.; Karremans, A.; Gonzalez, R.; Meneguzzo, T.; Kolanowska, M.; Tarazona, M.; Álvarez, L.; Dalström, S.; Dodson, C.; Fernández, M.; García, D.; Medina, H.; Mormontoy, R.; Nauray, W.; Rincón-Useche, C.; Ruíz, S.; Serguera, M.; Smith, C.; Villafuerte, M.; Vega, N. and F. Werner. (2015) Icones Orchidacearum Fascicle 14. The genus *Epidendrum*, Part 10: Species new and old in

Epidendrum. Herbarium AMO. Instituto Chinoin, A.C. Mexico City, 209 p. (only co-authored plates here shown - full monograph available at: http://www.herbarioamo.org/index_archivos/Fascicle14.pdf).

Own contribution (to 12 co-authored plates): Field work (60%); morphological analysis (including plates and illustration: 70%); manuscript preparation (60%).

Chapter V

Pérez-Escobar, O.; Balbuena, J.; and M. Gottschling (2016) *Syst. Biol.* 65: 51-65

Own contribution: Field work (80%); Laboratory work and sequence analysis (100%); *in-silico* work, phylogenetic analysis and scripting (50%); manuscript preparation (40%); images (100%).

Chapter VI

Pérez-Escobar, O.; Gottschling, M.; Whitten, M.; Salazar, G. and G. Gerlach (2016) *Mol. Phyl. Evol.* 97: 1-10

Own contribution: Field work (70%); Laboratory work and sequence analysis (100%); phylogenetic analysis (100%); manuscript preparation (40%); images (100%).

Chapter VII

Pérez-Escobar, O.; Gottschling, M. and G. Gerlach (*in revision*) *Journal of Biogeography*

Own contribution: Field work (70%); Laboratory work and sequence analysis (100%); phylogenetic analysis (100%); manuscript preparation (50%); images (100%).

Oscar Alejandro Pérez-Escobar (Signature)

Prof. Dr. Marc Gottschling (Signature)

ORAL PRESENTATIONS

PÉREZ-ESCOBAR, O. Phylogenetics and Biogeography of Darwin's favourite orchids. *9th Evolution, Ecology and Systematics Conference.* Munich, Germany, October 8th 2015.

PÉREZ-ESCOBAR, O. Quantifying divergent evolution between nuclear host and chloroplast endosymbionts. *Phylogeny Meets Genomics International Workshop.* Munich, Germany, May 11th 2015.

PÉREZ-ESCOBAR, O. How to assess divergent evolution between the nuclear host and chloroplast endosymbionts. *1st Programming for Evolutionary Biologist Conference.* Porto, Portugal, April 27th 2015.

POSTER PRESENTATIONS

PÉREZ-ESCOBAR, O.; VALDIVIESO, P. PARRA, E.; RINCÓN-USÉCHE, C. AND L.K. RODRÍGUEZ. Novelties in Orchidaceae for the Colombian Flora. *4th Scientific Conference on Andean Orchids*. Guayaquil, Ecuador, November 2nd, 2012.

PÉREZ-ESCOBAR, O.; GOTTSCHLING, M. AND J.A. BALBUENA. Quantification of phylogenetic incongruence between organellar and nuclear genomes. *17th Annual Meeting of the Gesellschaft für Biologische Systematik*. Munich, Germany, 21st February, 2016.

FIELD WORK

- Costa Rica: Punta Arenas, Alajuela, Limon. July 2013.
- Panama: El Valle de Anton, Coclé, Cerro Punta. August 2013.
- Nicaragua: San Juan del Sur, Boaco, Estelí, Managua. November 2014
- Mexico: Chiapas, Veracruz, Oaxaca. February 2015.

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SUMMARY

The Orchidaceae are one of the most species rich and widespread lineages among angiosperms. They have evolved numerous remarkable vegetative and reproductive traits that have allowed them to successfully adapt and diversify into a wide array of environments. More importantly, they have developed several intricate symbiotic relationships with different kinds of organisms (e.g. animals, fungi) that for centuries have attracted the attention of botanists, biologists, amateurs and naturalists. Nevertheless, despite the extensive research done so far on orchid biology and phylogenetics, very little is known about the biotic and environmental variables as well as the evolution of several key traits that seem to be linked with the successful diversification of this lineage. This dissertation is focused on three puzzling aspects of plant evolutionary biology, specifically the phylogenetic incongruence between nuclear and plastid genomes, the evolution of sexual systems, and lineage migration and isolation through time. To address these topics, I chose as a group of study the subtribe Catasetinae, an orchid lineage including ca. 350 species restricted to the Neotropical region. They show a remarkable set of sexual systems, namely protandry and Environmental Sex Determination (ESD), that were never studied before in a phylogenetic context. My dissertation includes as well a minor part on taxonomic and floristic work devoted to other representative orchid lineages of the Neotropical flora (i.e. *Epidendrum* and *Lepanthes*). Based on vegetal material collected during field trips, my taxonomic research resulted in the description of several new species and new chorological reports contributing to the Colombian and Costa Rican Floras.

Using a set of nuclear and chloroplast loci obtained from material cultivated at the Botanic Garden Munich and collected during field work in several Latin American countries, I produced a well-supported and insofar the most representatively sampled phylogeny of Catasetinae. While gathering vegetal material, I encountered several complications such as extreme scarcity of individuals and worrisome, extensive bureaucratic administrative processes to obtain collection and research permits that finally undermined my taxon sampling. By studying in detail the Catasetinae internal phylogenetic relationships independently derived from nuclear and plastid loci, I came across several well supported conflicting phylogenetic positions. Most of the traditional phylogenetic methods developed to address these conflicts aim at the inference of a species tree only. In chapter 5, I explored the utility of co-phylogenetic tools (i.e. PACo

and ParaFit) to quantify the conflicts between nuclear and plastid genomes. These tools have been largely employed in host-parasite/endosymbiont studies, hence they have the power to assess the contribution of single Operational Terminal Units (OTUs) to the phylogenetic pattern observed. As a result, using the Catasetinae chloroplast and nuclear datasets and extensive simulation approaches, I demonstrate that PACo successfully detects conflicting OTUs and its performance is overall better than ParaFit. In addition, my research provided strong evidence towards the bias of input data type (i.e. phylograms and cladograms) on distance-based co-phylogenetic methods. A pipeline to execute PACo and ParaFit tools in the software R to detect conflicting sequences in either small or big datasets was designed

After inferring a strongly supported phylogeny, and by carrying *in-situ* and *ex-situ* observations plus searches of specialized literature on reproductive biology, I investigated the evolution of sexual systems of Catasetinae. I relied on Ancestral State Reconstruction (ASR) approaches and Bayesian statistical frameworks (chapter 6). As a result, ASR revealed three independent gains of ESD, once in the Last Common Ancestor (LCA) of *Catasetum, Cycnoches* and part of *Mormodes*, respectively, always derived from a protandrous ancestors. In contrast, protandry appears to have evolved only once, at the LCA of *Catasetum*, *Clowesia*, *Cycnoches*, *Dressleria* and *Mormodes*.

The last chapter of this dissertation deals with the impact of the Andean uplift, the most important orographic event in South America, on evolution of epiphytic lowland Neotropical lineages. I used as a group of study *Cycnoches* (a member of the Catasetinae), which includes ca. 34 species and is distributed in Neotropical lowland wet forests. To address this goal, I produced the most completely sampled phylogeny of *Cycnoches*, and relied on Bayesian dating and Ancestral Area Estimation (AAE) approaches. The LCA of *Cycnoches* lived ca. 6 million years ago (MYA) in the Amazonian region. From this area, it expanded towards Central America and Choco in multiple migrations well after main Andean mountain building episodes. In addition, stochastic character mapping showed that within-region speciation (i.e. speciation in sympatric lineages) was a key process linked to diversification and range distribution evolution in *Cycnoches.*

Chapter 1

GENERAL INTRODUCTION

Biology of orchids

The orchid family (Orchidaceae) is one of the largest among flowering plants (Cribb et al., 2003). There is no consensus about the extant number of orchid species (Dressler, 1993), the Orchidaceae include about 25,000 species distributed in 736 genera (Chase et al., 2015; Givnish et al., 2015). To further complicate orchid diversity assessment, several new species and genera are described every year at an incredible pace (Padial & de la Riva, 2006; Chase et al., 2015), mostly by segregating species from monophyletic lineages into new genera and by using uninformative characters known to be extremely variable to propose new species. Within the angiosperm tree, orchids are placed as the sister group to all other members of the order Asparagales (Seberg et al., 2012), and their origin traces back to the late Cretaceous (~94 MYA, Chomicki et al., 2014a). Only three fossils are known for the orchid family, therefore absolute age estimation of orchid lineages is often challenging despite the unambiguous assignment of the few fossils to distantly related lineages (i.e. *Dendrobium* Sw., *Earina* Lindl.: 20-23 MYA, Conran et al., 2009; †*Meliorchis caribea*: 15-20 MYA, Ramírez et al., 2007),

Orchids are distributed everywhere across the Globe in terrestrial habitats (excluding the polar circles) (Pridgeon et al., 1999; Givnish et al., 2015), yet the greatest diversity is concentrated in tropical regions (Dressler, 1993). Orchidaceae are herbs mostly adapted to humid habitats but many have also evolved a wide array of morphological adaptations allowing them to survive in a great variety of ecosystems, including arid and semi-arid habitats (González-Tamayo, 2002; Trujillo & Rodriguez, 2011), and cold dry environments (e.g. Paramos: Chase, 1986). Orchid diversity distributed in temperate regions is often represented by terrestrial or lithophytic plants, whereas the majority of orchids occurring in tropical biomes are epiphytes (Pridgeon et al., 1999). Epiphytism is often regarded as the derived habit condition in orchids because of its appearance in the recent evolutionary history of tropical orchids (Givnish et al., 2015), and as a key innovation that promoted rapid adaptive radiations (Dodson, 2003).

Orchid plants usually have cylindrical or flattened roots that provide anchorage to the substrate (main orchid vegetative and reproductive structures are depicted in **Fig. 1**), but also serve as a photosynthetic, protective, water and nutrient absorption organs (Chomicki et al., 2014b). Those roots are often covered by an epidermal tissue called velamen, a structure predominantly present in epiphytic orchids (although it may occur

in some terrestrial taxa as well; Pridgeon et al., 1999). In terrestrial orchids, roots are often modified into storage organs (e.g. *Orchis* Tourn. ex L.) and instead of a velamen they have a simple rhizodermis provided with numerous radical trichomes. Orchid stems are classified as rhizomes and pseudobulbs (Dressler, 1993; Judd et al., 2007). The former term refers to a subterranean, horizontally growing stem that is embedded in the substrate whereas the latter is rather a thickened, modified stem that is exposed and serves as storage organ.

Orchid leaves resemble a traditional monocot leaf, with parallel venation (although few taxa present reticulate venation, e.g. *Epistephium* Kunth; Szlachetko et al., 2013), and they are either distichously or spirally arranged. Leaves in orchids perform photosynthesis (in the aphyllous orchid *Dendrophylax lindenii* (Lindl.) Benth. ex Rolfe it is performed by the roots, see Chomicki et al., 2014a) but also more specialized functions, such as pollinator attraction (i.e. *Phragmipedium* Rolfe: Ren et al., 2011). Perhaps one of the most fascinating aspect of orchids is the mesmerizing morphological diversity of their reproductive structures when compared with other monocot lineages. Orchid flowers are zygomorphic and generally consist of a set of three outer and three inner tepals, one of which is modified into a highly specialized structure called labellum (usually the median tepal of the inner whorl), and 1-3 stamens adnate to the style and stigma, forming a gynostemium (Bateman & Rudall, 2006; Judd et al., 2007; Mondragón-Palomino, 2013). Together with a 180° torsion of the flower during the development (i.e. resupination), the labellum and gynostemium are apomorphies that distinguish orchids from other monocot lineages (Judd et al., 2007). In most orchid species, the labellum is strongly ornamented (e.g. bearing appendages or calli) and plays a major role in pollination by serving as attractant and landing platform for pollinators (Darwin, 1877; Bateman & Rudall, 2006).

Orchid fruits (capsules) are composed by three carpels; they are loculicidal and dehisce at maturity. Fruits are usually green, photosynthetic during the development but at maturation, they turn yellow, usually being unattractive to animals. Seeds, sometimes called "dust seeds" because of their minute size (ranging from 8-10 µm to 5 mm in length), have usually a thin testae with a highly variable ornamentation that has been traditionally employed for classification (Chase & Pippen, 1990; Pridgeon et al., 1999; Barthlott et al., 2014). Most of the orchid seeds are anemorchorous, although in some

exceptional cases (i.e. *Cyrtosia* Blume), fruits are consumed by birds and hence ornithochorous (Suetsugu et al., 2015).

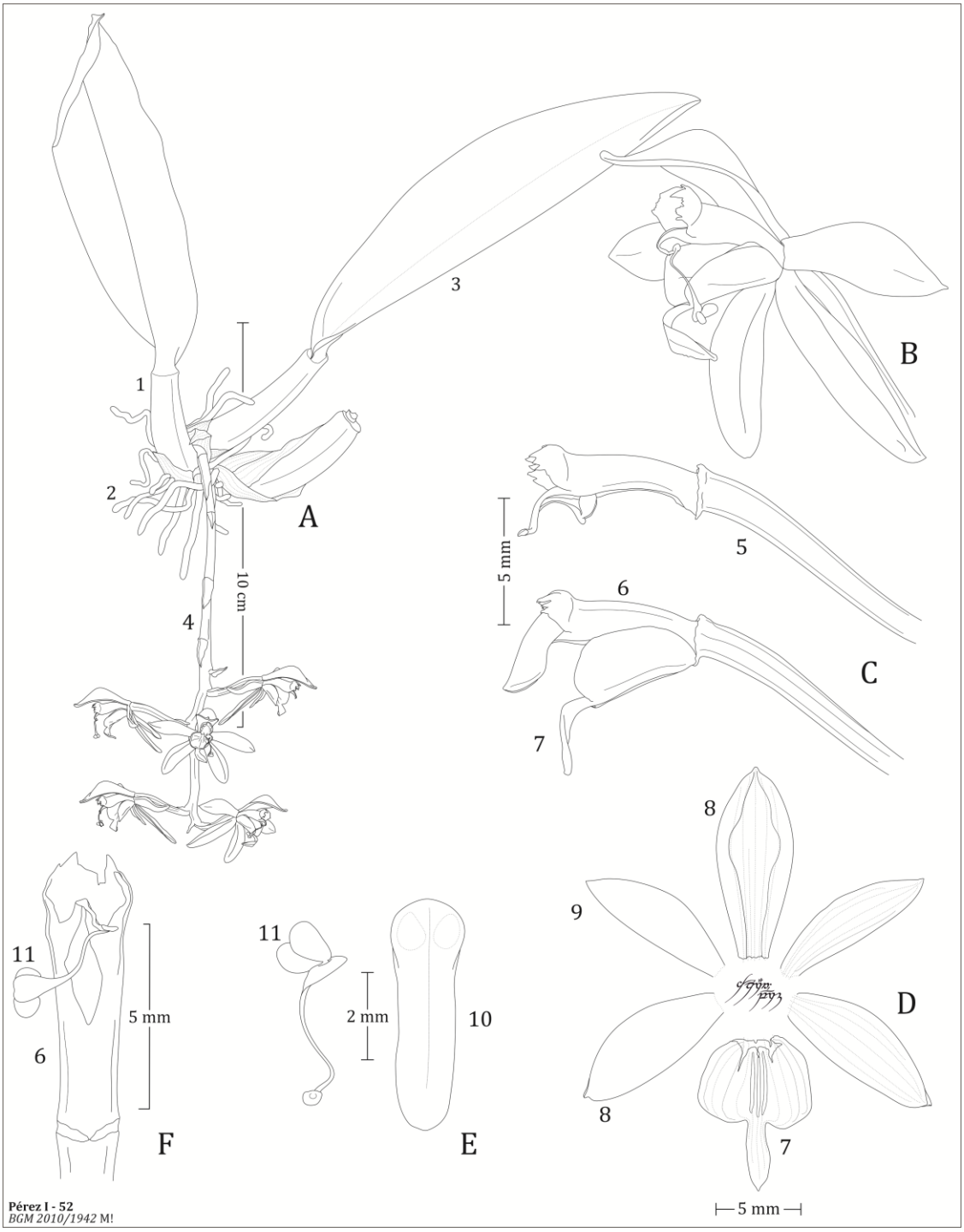


Figure 1. Schematic view of main orchid vegetative and reproductive structures. **A**. Plant: 1) Pseudobulb, 2) Root, 3) Leaf, 4) Inflorescence; **B.** Flower (3/4 view); **C.** Side view of ovary (5), column (6) and lip (7); **D.** Dissected flower: 8) outer tepals, 9) inner tepals; **E.** Pollinarium: 10) pollinia and 11) anther cap; **F.** Column (ventral view). Drawing by O. Pérez based on *BGM 2010/1942* M.

One peculiarity of orchid seeds is the lack of a nutritional tissue (endosperm and cotyledons – some rudiments of the latter still occurring in few taxa) (Dressler, 1993; Pridgeon et al., 1999). Instead, they have evolved mutualistic relationships with fungi, on which they rely during early stages of development (e.g. germination) for provision of major nutrients such as carbon (Cameron et al., 2006). These mutualistic associations (known as mycoheterotrophy) may persist throughout life history of some orchid species (e.g. achlorophyllous species such as *Dendrophylax lindenii*; Chomicki et al., 2014a). In other lineages however, mycoheterotrophy might not be life-lasting, as the vast majority of orchids produce green leaves and therefore are at least partially autotrophic (Cameron et al., 2006). The mode and tempo of evolution of this endosymbiosis is still elusive, but it might have appeared early in the history of orchids because it repeatedly occurs across all major orchid lineages, including early branching ones (e.g. Apostasioideae and Vanilloideae subfamilies; Warcup, 1981).

Contributions to the Neotropical Orchid Flora, and the challenge of working with tropical plants

Orchids are one of the most prominent components of Neotropical plant biodiversity, but also a common ornamental plant in both urban and rural settlements. The orchid growing and collection trace back to the botanical explorations commanded under colonial powers almost three centuries ago. Some of the most popular botanist explorers in the Neotropical region were Alexander von Humboldt (Humboldt, 1820), Jose Celestino Mutis (Royal Expedition of the New Granada Kingdom), and Carl Friedrich Phillip von Martius (Cogniaux et al., 1883). The legacy of such laborious work resulted in detailed monographs and catalogues of local and regional Floras (e.g. Humboldt, 1820; Cogniaux et al., 1883). Nowadays, several natural and anthropogenic variables pose new threats to natural orchid populations. Among these threats, habitat loss, indiscriminate orchid collection and smuggling are perhaps the factors that affect the most natural populations (Davenport & Ndangalasi, 2003; Neng, 2010). This is particularly true because the vast majority of orchid species are confined to limited geographic ranges and often, their populations comprise only few individuals (Cribb et al., 2003).

The imminent local and regional extinction of orchid populations urges botanists and ecologists to study in detail local and regional floras to gain essential knowledge on the abundance and richness of endangered species. Local and regional floristic inventories are key tools for biodiversity protection (Triana & Murillo, 2005) because they provide valuable insights on the conservation status (i.e. population abundance and distribution) of threatened species. Without the critical knowledge that these floristic treatments provide, governmental and private research institutions responsible for natural resources conservation hardly can propose measures to combat biodiversity loss. During my research work, I contributed to the knowledge of the Neotropical orchid flora with the description of several species previously unknown to science and with the report of chorological novelties as a result of intensive field work done in cloud forests of Central and South America (Andean region). These cloud forests are of great importance because they host a large part of the Neotropical orchid diversity (Orejuela-Gartner, 2012) and coincidentally, they are one of the most threatened biomes because of deforestation in several countries such as Colombia (Triana & Murillo, 2005). This taxonomic work was carried out in collaboration with leading researchers in orchid systematics (e.g. Eric Hágsater, Mexico) and was centred on two compelling orchid lineages: *Epidendrum* L. and *Lepanthes* Sw., which are an important component of the North Andean and Central American Montane Flora. This part of my research involved the study of major local and regional herbarium collections (e.g. HLDG, COL, CR, CUVC, JBL, VALLE), as well as plant material documentation (photographing, illustration). These floristic novelties were published as research articles in per-reviewed journals (**Chapter 2, 3**) or as contributed chapters to a monograph (**Chapter 4**).

Facing orchid local and regional population extinction, fortunately several international treaties and conventions controlling wildlife trade, its products and derivates have been established recently. Among the most famous worldwide treaties is the CITES (Convention on International Trade in Endangered Species of Wild Fauna and Flora). CITES is an international agreement signed insofar by ca. 160 member governments (Mulliken, 2009), whose aim is to ensure that international trade of wild animals and plants does not threaten their survival (www.cites.org). Although some authors have acknowledged the success of such treaty (e.g. Pritchard, 1989) on endangered orchid populations, there is still some controversy regarding the real impact of CITES on the conservation status of the orchid species included in their list (Mulliken,

2009). This is mostly because CITES operates in conjunction with several other local, national and international regulatory processes, some of which are subject to large and unfruitful bureaucratic procedures. Traditionally, to export orchid material, several certifications are required such as field collection, phytosanitary and export permits (Pritchard, 1989). Nevertheless, to obtain these permits sometimes represent a tedious, time expensive process for the researcher that is likely to result in a delayed expedition of the required documentation and sometimes in a loss of valuable material for study.

The focus of my dissertation project was centred on Neotropical orchids, all of which are often scarce in herbarium collections. Consequently, I relied mostly on plant material that I collected in the field, facing then several complications regarding collection and export permits issuing in some of the countries where I carried field trips (e.g. Nicaragua). More importantly, I was unable to get vegetal material in few other countries (e.g. Colombia and Ecuador) simply because regulations to get the required certifications are too stringent and time-consuming. To some extent, this limited my research goals because some of them were directly dependent on the availability of material from orchid species with very narrow distribution. However, thanks to the collaborative effort held between European botanical gardens (e.g. Botanischer Garten Heildelberg, Botanischer Garten München), valuable missing material was obtained from other living collection via garden exchanges.

Diversity and distribution of the subtribe Catasetinae

"I have reserved for separate description one sub-family of the Vandeae, namely the Catasetidae, which may, I think, be considered as the most remarkable of all Orchids"
(Darwin, 1877, p. 211)

The Neotropics are one the most biodiversity rich regions on Earth, harbouring about 90,000 – 110,000 seed plant species (Antonelli & Sanmartín, 2011). Among the angiosperm lineages distributed in the Neotropics with the highest degree of endemism and diversity are the orchids (Gentry & Dodson, 1987). Within such extraordinary diversity, the subtribe Catasetinae is an important component of the Neotropical flora (Funk et al., 2007), but also a remarkable lineage because of their peculiar reproductive biology (see section on sexual systems of this *Introduction*; Romero, 1990). The Catasetinae comprise approximately 350 species that are distributed in tropical and

subtropical regions of America (**Fig. 2**; Romero, 2009) and are classified in eight genera, namely *Catasetum* Rich. ex Kunth, *Clowesia* Lindl., *Cyanaeorchis* Barb.Rodr., *Cycnoches* Lindl., *Dressleria* Dodson, *Galeandra* Lindl., *Grobya* Lindl., and *Mormodes* Lindl. (Chase et al., 2015) (see **Fig. 3**).

Catasetum, the species richest lineage in the subtribe, comprises 170 epiphyte species including several natural hybrids [e.g. *Catasetum* x *roseoalbum* (Hook.) Lindl.; Romero & Jenny, 2009]. *Catasetum* is widely distributed from Mexico to Southern Brazil and Argentina, although its centre of diversity is the Amazonian forest of Brazil (Pridgeon et al., 2009). *Mormodes* and *Cycnoches*, with 80 and 34 epiphyte species respectively, have similar distribution ranges and habitat preferences as *Catasetum*, and are best represented in Central America (Sosa & Rodríguez-Angulo, 2000; Pridgeon et al., 2009) and in the Amazonian region, respectively (Pridgeon et al., 2009; Carr, 2012). *Galeandra*, which includes 38 species, is the sole genus of the Catasetinae with both epiphytes and geophytes, the latter living in lowland gallery forests, savannas and humid areas. It has a wider distribution than *Catasetum* and *Cycnoches*, ranging from Southern Florida to Southern Brazil and Argentina, but like for those lineages, the vast majority of its diversity is found in Brazil (Pridgeon et al., 2009; Monteiro et al., 2010).

The remaining lineages (i.e. *Clowesia*, *Dressleria*, *Grobya* and *Cyanaeorchis*) have much narrower distribution ranges. *Dressleria*, for instance, includes 11 species distributed from Nicaragua to Peru. Rather than being represented in lowland tropical forests like most of the Catasetinae species, it is restricted to the cloud forest' understory at mid to high elevations in the Andes (Dodson, 1975; Pridgeon et al., 2009). *Clowesia*, a clade with seven species, is distributed from Mexico to Ecuador. Plants of this small clade commonly live in tropical lowland wet forests, and its diversity is concentrated in Mexico (Dodson, 1975). *Grobya* comprises five epiphytic species restricted to southeastern Brazil. They are mostly found in Brazilian wet forests (Mata Atlantica) and rocky outcrops (Campos Rupestres) (Barros & Lourenço, 2004; Pridgeon et al., 2009). Finally, *Cyanaeorchis*, the smallest clade of the Catasetinae with three geophytic species, is restricted to northeastern Brazil, Argentina and Paraguay. Like in some species of *Galeandra*, plants of *Cyanaeorchis* are often found growing in humid grasslands and marshes (Batista et al., 2014).

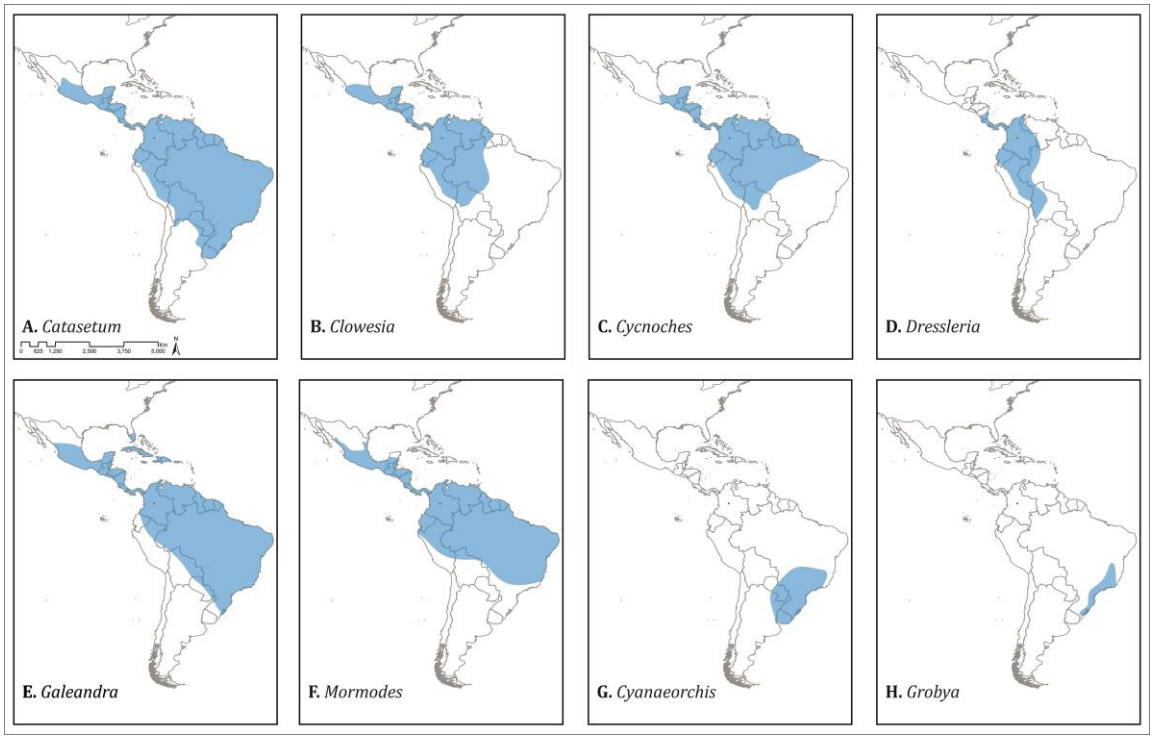


Figure 2. Geographical range distribution of Catasetinae genera.

Figure 3. Representative species of Catasetinae genera. **A**. *Catasetum cernuum* (Lindl.) Rchb.f.; **B**. *Clowesia russelliana* (Hook.) Dodson; **C**. *Cyanaeorchis arundinae* (Rchb.f.) Barb.Rodr.; D. *Cycnoches rossianum* Rolfe; **E**. *Dressleria dilecta* (Rchb.f.) Dodson; **F**. *Galeandra leptoceras* Schltr.; **G**. *Grobya* sp.; **H**. *Mormodes ephippilabia* Fowlie. Pictures: O. Pérez, G. Gerlach & L. Villez

Taxonomic history and molecular phylogenetic relationships of Catasetinae

Recent discoveries of new species and expeditions to previously inaccessible areas made available material that was unreachable before. Hence taxonomic work on Catasetinae was done, leading to several changes on its circumscription during the last century. Of special interest is the generic delimitation of some of its members, which has been "fluctuating" during the last decades. The Catasetinae were erected by Lindley (1843) under the name of Catasetidae, and five genera were established: *Catasetum, Clowesia, Cycnoches, Cyrtopodium* R.Br. in W.T.Aiton and *Mormodes*. Almost 40 years later, Bentham (1881) transferred *Catasetum*, *Cycnoches,* and *Mormodes* to Stanhopeinae, another prominent Neotropical subtribe. Most botanists however, endorsed Lindley's Catasetinae concept, including *Catasetum, Mormodes,* and *Cycnoches,* but also *Clowesia* (segregated from *Catasetum*) and *Dressleria* (Dodson, 1975), all forming the so called "core Catasetinae". *Cyrtopodium* in contrast, has been since then assigned to different subtribes (i.e. Cyrtopodiinae and Cymbidiinae) based on cladistic and phylogenetic inferences using anatomical characters (Stern and Judd, 2001) and nucleotide sequences (Whitten et al., 2014; Givnish et al., 2015), respectively. Therefore, *Cyrtopodium* will not be considered here as a member of Catasetinae because their phylogenetic placement is still a matter of debate.

The advent of cladistic and molecular phylogenetic approaches brought new insights into the systematics and evolution of Catasetinae. The seminal works of Romero (1990) and Stern and Judd (2001), based on cladistic inferences from morphological and histological characters, supported the monophyly of Catasetinae *sensu* Dressler (1975) (i.e. core Catasetinae) by the presence of sunken foliar trichomes and the clinandrium antennae, a structure responsible for pollinarium ejection. Based on 30 morphological characters (of which 10 were informative), Romero (1990) placed *Catasetum* as sister group to the remainder of the core Catasetinae, and *Clowesia* was recovered as sister lineage of the clade *Dressleria* + (*Cycnoches* + *Mormodes*). Similar relationships were recovered by Stern and Judd (2001), with *Cycnoches* + *Mormodes* found as sister clade of the polytomy *Dressleria-Clowesia-Catasetum*.

More recent studies based on DNA sequences have provided support for the monophyly of the core Catasetinae (Pridgeon & Chase, 1998) as well. More importantly, they have endorsed the inclusion of *Cyanaeorchis, Galeandra* and *Grobya* in

Catasetinae, previously assigned to four different subtribes (Chase et al., 2003; Freudenstein et al., 2004; Pridgeon et al., 2009; Batista et al., 2014) reflecting the lack of morphological cohesion within Catasetinae (Pridgeon et al., 2009). Using nuclear ITS and mitochondrial *rps*4 sequences, Pridgeon and Chase (1998) reconstructed *Catasetum* as sister group to *Clowesia* in a rather derived, strongly supported clade. The clade *Dressleria* + (*Mormodes* + *Cycnoches*) was in turn placed as sister to *Catasetum* + *Clowesia*, albeit in a moderately supported clade. Freundenstein et al. (2004) placed *Galeandra* and *Grobya* within Catasetinae as sister lineages to the core Catasetinae based on a Maximum Parsimony tree inferred from a concatenated *mat*K-*rbc*L chloroplast dataset. Although the monophyly of the newly circumscribed Catasetinae received maximal statistical support, internal phylogenetic relationships were not strongly supported. Batista et al. (2014), using combined nuclear ITS and chloroplast *mat*K, *trn*K and *rbc*L loci, later included *Cyanaeorchis* in their Catasetinae phylogeny and found it as sister group to *Grobya* in a strongly supported clade. This clade in turn was recovered as sister group to the remaining lineages of Catasetinae (*Galeandra*+(*Catasetum*+(*Cycnoches*+*Dressleria*))). All those studies however only included a limited taxon sample of the extant species richness, usually one or two taxa for each genus (Batista et al., 2014; Whitten et al., 2014; Freudenstein & Chase, 2015; Givnish et al., 2015), making it impossible to disentangle evolutionary relationships between species (Pridgeon et al., 2009).

Phylogenetic incongruence between nuclear and chloroplast DNA datasets

Understanding evolutionary relationships between organisms, genes, or molecules is a central question of evolutionary biology, with the phylogenetic tree playing an important role as a tool for analysis and depiction (Barraclough & Nee, 2001; Choi & Gomez, 2009). Nevertheless, inferring phylogenies of plant lineages is challenging, because the phylogenies independently derived from nuclear and chloroplast DNA sequences often reveal conflicting relationships (Hardig et al., 2000; Kim & Donoghue, 2008). During the past two decades, an astonishing number of research works reporting discordance between nuclear and chloroplast phylogenies in several plant lineages have been published (e.g. Araceae: Nauheimer et al., 2012; Asteraceae: Fehrer et al., 2007; Orchidaceae: van der Niet & Peter Linder, 2008;

Saxifragaceae: Soltis & Kuzoff, 1995), indicating how frequent is this phenomenon in angiosperms.

Several factors have been acknowledged as potential causes of phylogenetic incongruence, such as non-biological artefacts (e.g. taxon sampling error, long branch attraction: van der Niet & Peter Linder, 2008), and biological processes such as Incomplete Lineage Sorting (ILS), chloroplast capture via hybridization and Horizontal Gene Transfer (HGT) (Rieseberg & Soltis, 1991; Soltis & Kuzoff, 1995; Fehrer et al., 2007). Altogether they produce to some extent discordance between phylogenies, yet their relevance is dependent upon the lineage of interest, the molecule or DNA loci used for phylogenetic inference, the statistical support of the discordance and the process associated with the incongruence (Soltis & Kuzoff, 1995). I encountered several conflicting positions between independently derived nuclear and chloroplast phylogenies while investigating the internal phylogenetic relationships of Catasetinae. Unlike phylogenetic discordances reported insofar in other plant lineages (e.g. Nauheimer et al., 2012; van der Niet et al., 2013), which are often not statistically **s**upported, those in Catasetinae were recovered with high to maximal statistical support (**Chapter 5** of this dissertation).

A battery of techniques to handle incongruences between phylogenies are already available, and they have undergone major developments during the last decade (Choi & Gomez, 2009; de Vienne et al., 2012). These comparative methods have two main goals: 1) quantify the incongruence or degree of difference between the datasets, and 2) infer a species tree from a set of incongruent trees (i.e. derived from genes or genomes), irrespectively of the biological process responsible for the incongruence (e.g. Kubatko et al., 2009; Larget et al., 2010; Liu et al., 2010). These methods have proven to be useful when species tree inference from conflicting datasets is desired, but their applicability is limited when the goal is to assess the contribution of specific Operational Terminal Units (OTU). For instance, these methods can reliably define the proportion of gene trees that support a given topology among a gene tree dataset (e.g BUCKy: Larget et al., 2010). However, they do not have the capability to assess the contribution of single OTUs to the observed phylogenetic pattern nor to determine the proportion of associations (i.e. any linked pair of OTUs in two phylogenies) that are conflicting among the tree dataset. Far from being an "obstacle" to evolutionary relationships inference, conflicting tree associations are of great interest because they often provide valuable information on

biological processes responsible of incongruences (e.g. HGT, ILS) (de Vienne et al., 2012).

Distance based co-phylogenetic approaches are comparative methods which employ distance matrices (e.g. patristic distances) to introduce phylogenetic information into a statistical framework (de Vienne et al., 2011). Based on the premise that changes in relationships of coevolving systems are reciprocally dependent and therefore result in topological similarity (Choi & Gomez, 2009; Balbuena et al., 2013), these methods have been largely applied to investigate co-phylogenetic structures as observed in host – parasite / endosymbiont systems (e.g. pocket gopher-cehweing lices: Legendre et al. 2002; Monogenea-fishes: Simková et al., 2004; papilloma viruses-vertebrates: Gottschling et al., 2011). They have the power to determine similarities between sets of trees, and to assess the contribution of a particular set of OTUs (i.e. associations) to the phylogenetic pattern observed. Chloroplasts, which nowadays are recognized as organelles of endosymbiotic origin derived from free living cyanobacteria (Mereschkowsky, 1910; Margulis, 1993), are dependent onto the nucleated host cell. Hence, the evolutionary history of the chloroplast endosymbiont genome is expected to track that of the nuclear host cell. By applying the sample principle of parasite-host dependence employed by the above-cited co-phylogenetic methods to that of chloroplast endosymbiont-nucleated host cell, detection of conflicting associations on a statistical framework between derived chloroplast and nuclear trees is made possible.

Reproductive systems in Catasetinae

Perhaps the most striking traits of Catasetinae are the sexual systems and pollination syndromes they have evolved. They make this lineage an appealing group and hence it has received much attention from botanists, orchid growers, amateurs, and naturalists including Darwin himself. As a rule of thumb, orchids are monogamous and produce bisexual flowers, either dichogamous (i.e. with temporal separation of male and female reproductive structures) or adichogamous (i.e. no temporal separation of sexes) (Dressler, 1993). In Catasetinae however, some species produce unisexual flowers, and protandry and Environmental Sex Determination (henceforth referred to ESD) are the two predominant sexual systems whereas adichogamy occurs in a small number of species only. Protandry, defined as a form of dichogamy with earlier maturation of the

staminate structures in unisexual and bisexual flowers, is a widespread sexual system in angiosperms (De Jong et al., 2011; Renner, 2014). In orchids, it has independently evolved multiple times across distantly related lineages (e.g. Catasetinae, Stanhopeinae, Cranichidinae, Goodyerinae, Spiranthinae) (Ackerman, 1977; Singer & Sazima, 2001; Jersáková & Johnson, 2007). Within Catasetinae, protandry occurs in *Dressleria*, *Clowesia* and in some species of *Mormodes*. In these lineages, the pollinarium (which blocks the stigmatic chamber entrance – see Fig.1 of **Chapter 6**) must be removed before the pollinia can be deposited in the stigmatic chamber.

Unlike protandry, ESD is an exceedingly rare sexual system, occurring in ca. 250 species of angiosperms only (Renner, 2014). Environmental sex determination, also known as "sex disphasy" or "plasticity" (Korpelainen, 1998; Renner, 2014) describes flexible changes of sex expression in response to (and entirely determined by) environmental variables such as type of substrate and sunlight photoperiod during an individual's life span (Schlessman, 1988; Korpelainen, 1998). In orchids, ESD exclusively occurs in all species of *Catasetum* and *Cycnoches*, as well as in those species of *Mormodes* which not evolved protandry (Pridgeon et al., 2009). In these lineages, plants produce sexually dimorphic, functionally staminate or pistillate unisexual flowers in separate plants mostly, although intermixed inflorescences with flowers of both sexes are seldom produced (Fig. 4; Dressler, 1993; Gerlach, 2007; Pridgeon et al., 2009). Like in other plant lineages with ESD (e.g. Cucurbitaceae: Malepszy & Niemirowicz-Szczytt, 1991; Krupnick et al., 2000; Boualem et al., 2015), enhanced ethylene production depending on the amount of light received by the plant is likely to regulate the production of flower sex in Catasetinae. Consequently, plants exposed to longer photoperiods will produce pistillate flowers, whereas those with restricted access to sunlight are likely to produce staminate flowers (Gregg, 1982, 1983; Zimmerman, 2011).

Despite the relatively frequent occurrence of protandry in angiosperms and orchids overall, no single study has addressed the evolution of this sexual system using phylogenetic tools. Likewise, for ESD only two pioneer studies have addressed the ESD mode of evolution in two angiosperm lineages, namely Siparunaceae and *Acer* (Renner & Won, 2001; Renner et al., 2007). This is perhaps because of the lack of densely sampled phylogenies and dedicated field work and observations on sexual systems occurrence. For the particular case of Catasetinae, based on a cladogram inferred from morphological characters (for details see "Taxonomic history of Catasetinae" section of

this *Introduction*), Romero (1990) found that protandry and ESD (referred as unisexuality in Romero's work) were equally likely (based on the parsimony principle) to be the ancestral condition of core Catasetinae. Nevertheless, Romero's hypothetical evolutionary scenarios and the distribution of protandry and ESD in Catasetinae has remained elusive because of the lack of a well resolved, supported phylogeny.

Historical biogeography, molecular phylogenetics and species delimitations in the genus *Cycnoches*

Among the peculiar generic lineages of Catasetinae, *Cycnoches* is one of the most striking clades because of the remarkable sexual dimorphism of its unisexual flowers (a detailed description of sexual systems is provided in **Chapter 6** – see also **Fig. 4**). *Cycnoches* comprises 34 species distributed from Southern Mexico to Northern Brazil and Bolivia (**Fig. 3**) (Pridgeon et al., 2009; Carr, 2012). The highest species diversity occurs in the Amazonian region of Bolivia, Brazil and Peru. Plants of *Cycnoches* are epiphytes living in lowland wet forests from 0 to 800 m., mostly on trunks of dead trees. They are characterized by having a pseudobulb with multiple internodes, alternate, distichous leaves, lateral, arched inflorescences, and functionally unisexual flowers. A schematic representation of a typical plant of *Cycnoches* is provided in **Figs. 5** and **6**.

Cycnoches was erected by John Lindley (Lindley, 1843), using as a type specimen an Amazonian plant of *Cycnoches loddigesii*. About half a century later, Rolfe (1909) subdivided the genus into two sections: *i*) sect. *Cycnoches*, which includes species with similar functionally pistillate and staminate flowers, and a ventricose lip with entire margin (**Fig. 5**); and *ii*) sect. *Heteranthae*, consisting of species with markedly different functionally pistillate and staminate flowers and a lip bearing dactylar processes (**Fig. 6**). Since then, only one major taxonomic revision of the genus has been published (Allen, 1952), which endorsed Rolfe's infrageneric classification. The taxonomy of *Cycnoches* has thus remained unattended until recently, after several new species have been described from the Amazonian forests of Bolivia (Carr, 2012).

Figure 4. Functional, sexually dimorphic pistillate (A) and staminate (B) flowers of *Cycnoches ventricosum* Bateman. Intermixed pistillate and staminate flowers in a single inflorescence of *C.* aff. *powellii* Schltr. (C) and *C.*aff. *pachydactylon* Schltr (D). Pictures by O.Pérez and G.Gerlach.

Previous molecular phylogenetic studies involving *Cycnoches* (e.g. Pridgeon & Chase, 1998; Freudenstein et al., 2004; Freudenstein & Chase, 2015) rather focused on other genera of Catasetinae (i.e. *Cyanaeorchis*: Batista et al., 2014; *Galeandra*: Monteiro et al., 2010) and included no more than three species of the genus. Therefore, neither the internal phylogenetic relationships of *Cycnoches* nor the monophyly of sections *Cycnoches* and *Heteranthae* were reliably understood before my research work. The lack of a solid phylogenetic framework has precluded further research on several evolutionary aspects of *Cycnoches*, such as the biogeographical history and evolution of sexual dimorphism within this lineage.

Figure 5. Schematic view of a member of *Cycnoches* sect. *Cycnoches* (*C. ventricosum* Bateman). **A**. Plant; **B**. Flowers (side view); **C**. Column (transversal cut) of a staminate flower: 1) non-functional stigmatic chamber, 2) rostellum band, 3) clinandrium, 3a) projections, 3b) filament; **D**. Lip: 4) claw, 5) calli, 6) hypochile, 7) epichile; **E**. Pollinarium: 8) pollinia, 9) stipe; 10) viscidium. Drawing by O. Pérez based on *BGM 2003/3993* M.

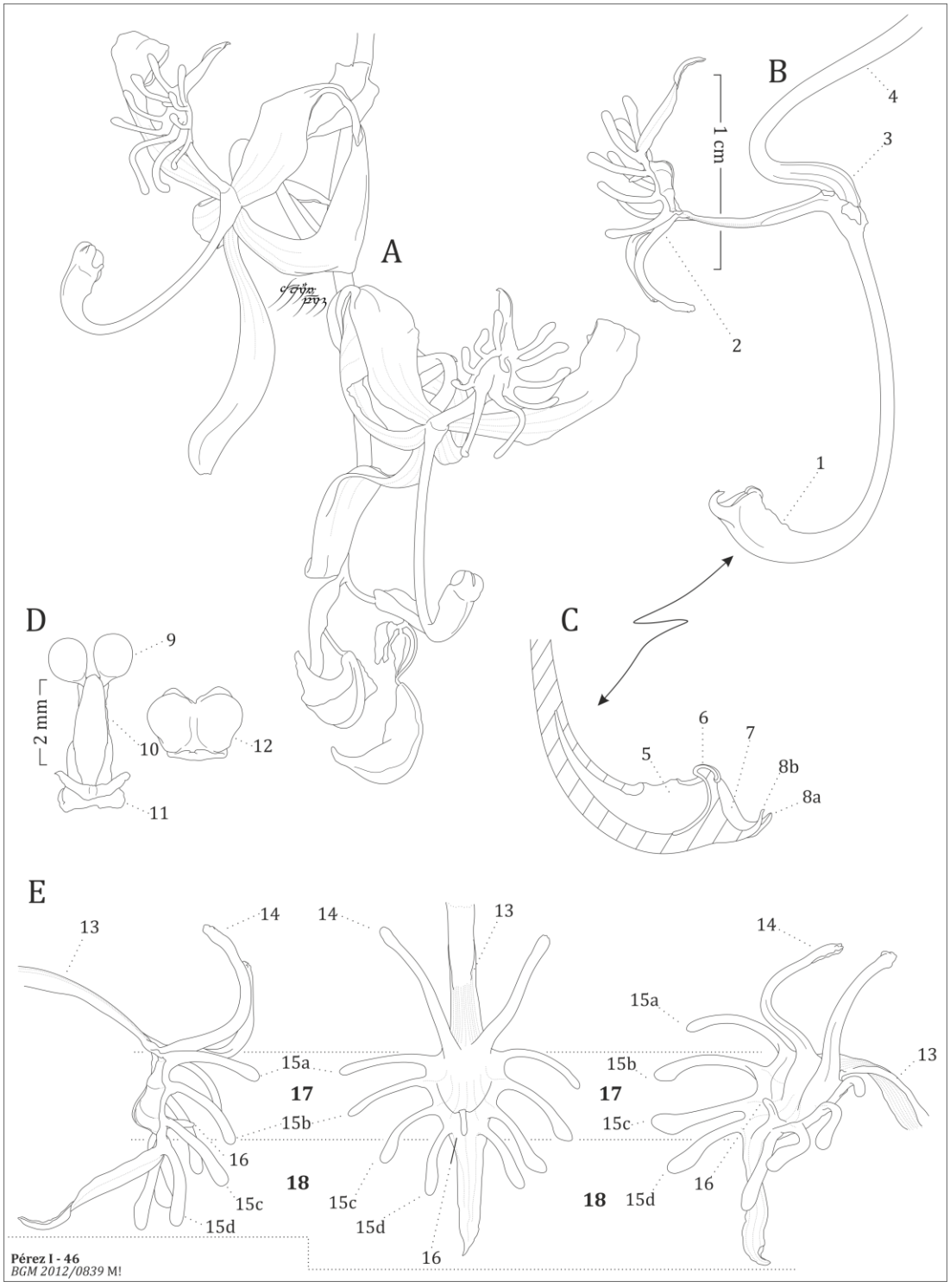


Figure 6. Schematic view of a member of *Cycnoches* sect. *Heteranthae*. **A.** Flowers; **B**. Side view of column (1), lip (2), ovary (3) and 4) pedicel; **C**. Column (transversal cut) of a staminate flower: 5) non-functional stigmatic chamber, 6) rostellum band, 7) clinandrium, 8a) projections, 8b) filament; **D**. Pollinarium: 9) pollinia, 10) stipe, 11) viscidium, 12) anther cap; **E**. Lip: 13) claw, 14) calli, 15a-15d) dactylar processes, 16) apical callus, 17) hypochile, 18) epichile. Drawing by O. Pérez based on *BGM 2012/0839* M.

Another puzzling aspect of *Cycnoches* is the extreme morphological variability of the reproductive structures occurring in some of the species (Gregg, 1983; Gerlach & Pérez-Escobar, 2014). This is reflected in the particular intricacy of the taxonomy in a group of species, denoted as the "*Cycnoches egertonianum* complex" (Romero and Gerlach, *in press*), which includes 10 entities (**Appendix S1**) distributed from southern Mexico to Southern Panama that are often difficult to identify from herbarium specimens. One peculiarity of all members from such complex is the large intraspecific variability of the lip dactylar processes (**Fig. 6**). For instance, Gregg (1983) reported for eight individuals of *C. dianae* Rchb. f (a member of *C. egertonianum* complex; **Fig. 7**) contrastingly different colour, forms and dactylar processes morphology, ranging from pink, rounded, very short to yellow-tan, oblong processes. Hence morphology does not provide useful information to delimitate species in the *C. egertonianum* complex. However, analysis of fragrances produced by Euglossine bee pollinated orchids such as *Cycnoches* is a powerful tool to carry on species delimitation because these fragrances are quite specific and often attract a single pollinator or a set of unique pollinators (Williams & Whitten, 1983). In addition, genome restriction-site-associated markers are a powerful approach to study genome divergences and address evolutionary questions at a population level because of the large number of reads of potential homologous loci it produces to perform comparisons between individuals (Eaton, 2014).

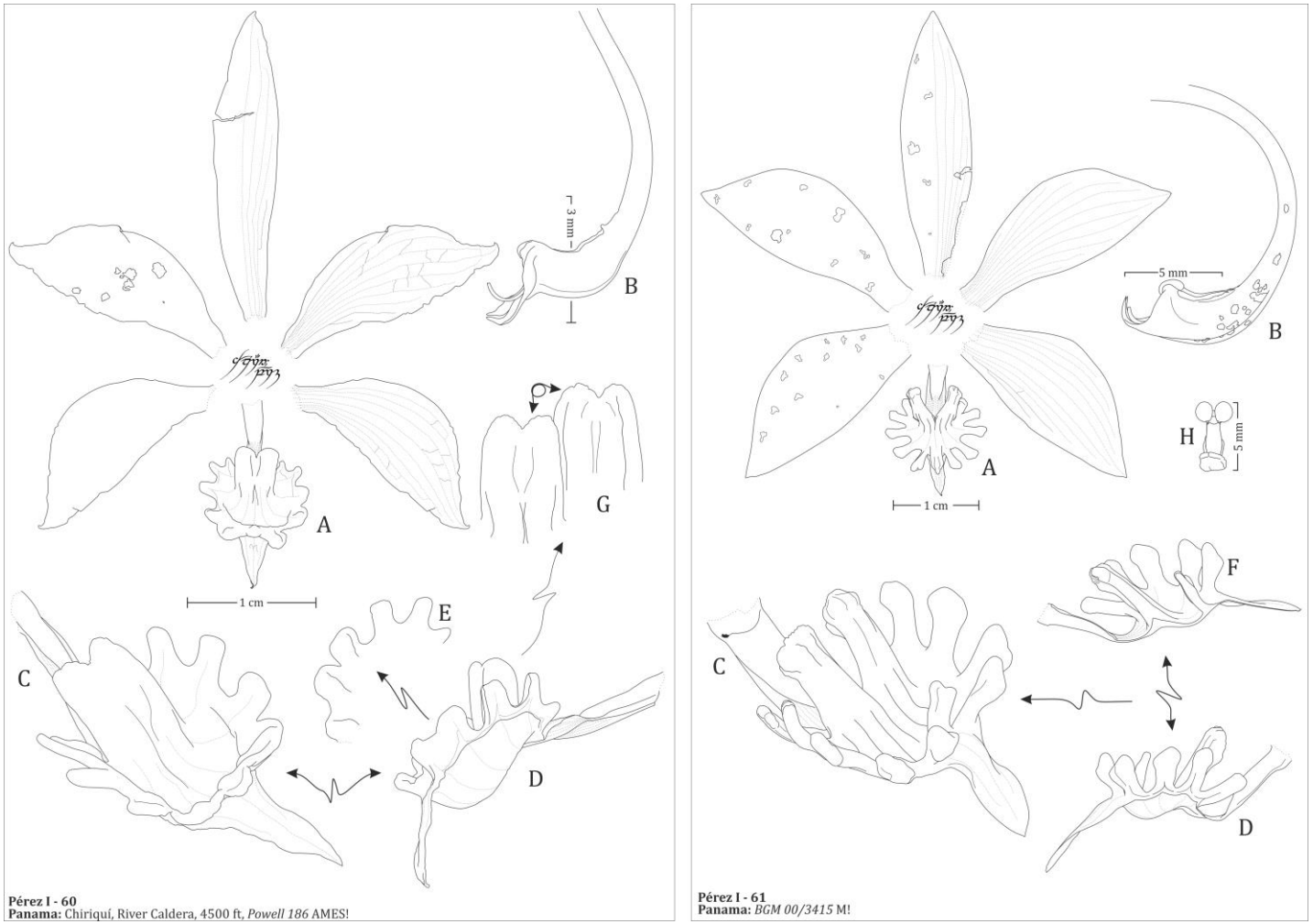
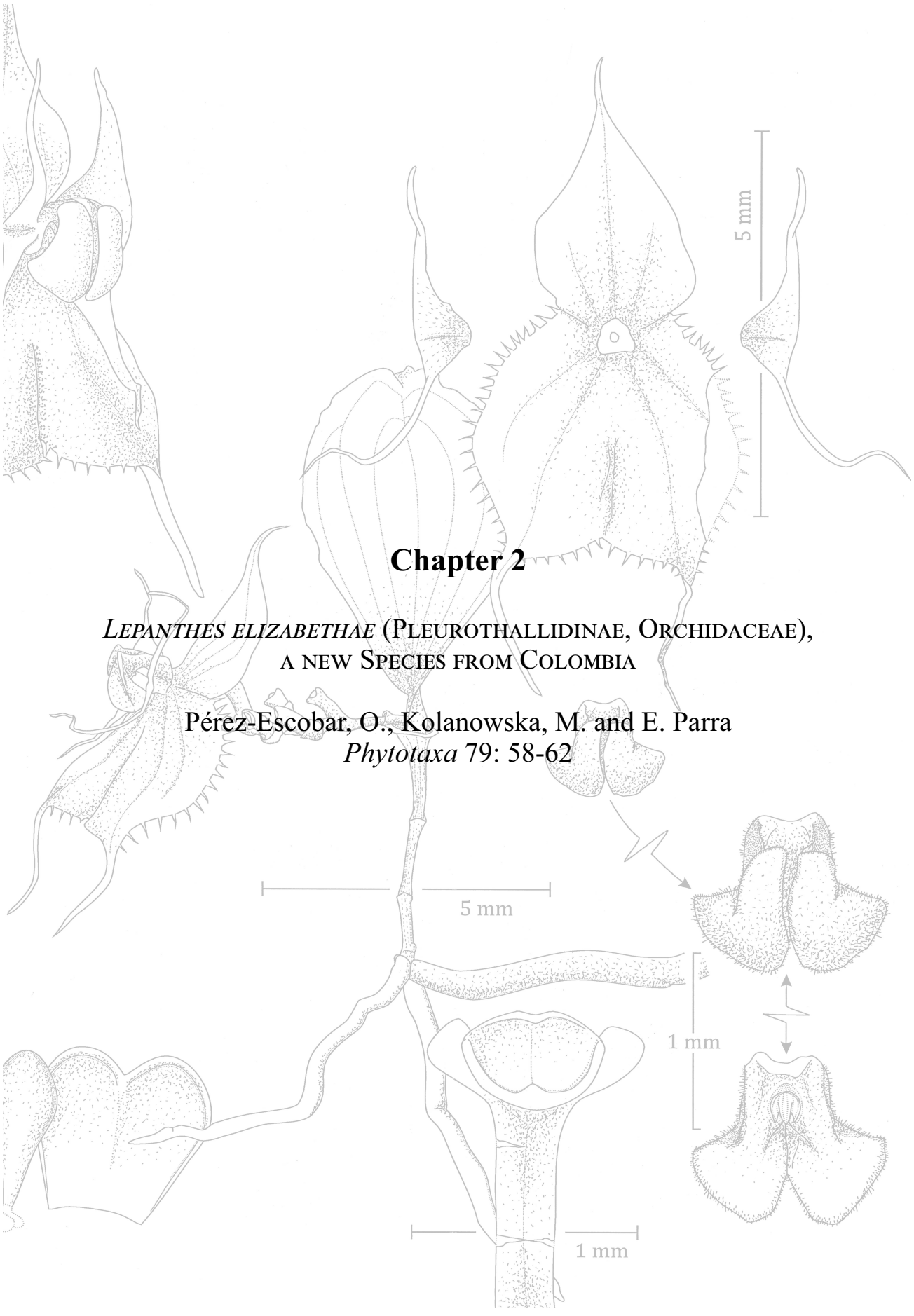


Figure 7. Schematic view of two flowers of *Cycnoches dianae*, drawn from different individuals. Note the different morphology of the dactylar processes in the labellum of both flowers. A. Dissected flower; B. Column (side view); C. Lip (3/4 view); D. Lip (side view); E. Detail of the dactylar processes; F. Lip (transversal cut). Drawing by O. Pérez based on *Powell 186* AMES (left) and on *BGM 00/3415* M (right).

Aim of the thesis

The main goal of my research was to better understand the role of biotic and abiotic factors on Neotropical orchid evolution by investigating the molecular phylogenetics, historical biogeography and trait evolution in Catasetinae and *Cycnoches* orchid lineages. In addition, because morphology does not provide useful information to delimitate species in the *C. egertonianum* complex, I investigated floral fragrance composition and genome divergence using Next Generation Sequencing data to better understand species boundaries in this complex. Furthermore, I aimed to explore the utility of two distance based co-phylogenetic tools, namely PACo (Procrustes Application to Cophylogeny – PACo: Balbuena et al., 2013) and ParaFit (Legendre et al., 2002) to detect conflicting sequences in independently derived nuclear and chloroplast phylogenies of Catasetinae. In particular, the main questions I addressed were: *i*) how many times did sexual dimorphism evolved in Catasetinae? *ii*) did the LCA of Catasetinae bore bisexual, protandrous flowers? *iii*) when and where did the LCA of *Cycnoches* diversify? *iv*) Did the Andean uplift represent an isolative barrier for lowland epiphytic lineages such as *Cycnoches*?

To answer these questions, I compiled a more comprehensive, densely sampled molecular dataset of Catasetinae, from which I produced the most representatively sampled phylogeny of Catasetinae so far published (**Chapters 5 and 6** of this thesis). Based on this new solid phylogenetic framework, I addressed the evolution of sexual systems by gathering information on mating system data and performing ASR using different approaches (Maximum Likelihood and Bayesian methods; **Chapter 6**). To quantify the utility of PACo and ParaFit tools to retrieve conflicting sequences, I analysed the Catasetinae nuclear and chloroplast derived trees as well as simulated datasets, which lately provided a solid statistical testing framework of these applications under different data conditions (**Chapter 5**). I also developed a pipe-line in cooperation with Dr. Juan Balbuena (University of Valencia) to automatize the outlier detection process and apply it to any set of trees (i.e. either large or small datasets – 50 to 200 OTUs). Finally, to determine the role of the Andean uplift into geographic range evolution of lowland epiphytic lineages, I investigated the biogeographical history of *Cycnoches* using a well-resolved, novel chronogram and modern phylogenetic approaches (**Chapter7**).



Chapter 2

Lepanthes elizabethae (Pleurothallidinae, Orchidaceae), a new Species from Colombia

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Lepanthes elizabethae (Pleurothallidinae, Orchidaceae), a new species from Colombia

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Introduction

With over 800 species, *Lepanthes* Swartz (1799: 85) is one of the largest genera in Pleurothallidinae (Orchidaceae). It ranges from southern Mexico to Bolivia and northern Brazil. A high level of endemism is observed in the Andes of Colombia and Ecuador (Pridgeon 2005). Despite the large number of species described by Luer (1986, 1994, 1996, 2009), several new *Lepanthes* have been described by other authors (Catling & Catling 1988, Tremblay & Ackerman 1993, Ortiz 1998, Pupulin & Bogarín 2004, Pupulin *et al*. 2010).

Plants of *Lepanthes* usually grow epiphytically in cloud forests and paramos, but some on rocks and the ground have also been reported (Farfán *et al*. 2003). Species of *Lepanthes* are easily recognized by their lepanthiform sheaths, successive inflorescences arising from the upper- or underside of the leaf, usually transversely bilobed petals and often bilobed lip (Farfán *et al*. 2003), usually with an appendix attached to the sinus of the body. Inflorescences are rarely simultaneous, as in *L. foreroi* P.Ortiz, O.Pérez & E.Sánchez (2009: 137) and *L. pleurorachis* Luer (1983: 363). The greatest species diversity is found in Colombia with 305 species (Vieira pers. com). New taxa and unreported species for the Colombian flora are described and published every year (Ortiz *et al*. 2009, 2010, Calderón 2010, Pérez *et al*. 2010).

During field studies conducted in the department of Valle del Cauca, a new *Lepanthes* was found. It resembles *L. lycocephala* Luer & Escobar (1984: 147), from which it differs by the plant size and shape of upper lobes of petals, lip blades and lip appendix.

Lepanthes elizabethae* O.Pérez, Kolan & E.Parra, *sp. nov. (Figs. 1, 2)

Type:—COLOMBIA. Valle del Cauca: Municipio de Yumbo, Corregimiento de DAPA, Bosque de Niebla residual entre las fincas Cielo Azul y DEBUSALE, ca. 1800 m, 10 October 2010, *Pérez, González & Buβ 999* (holotype CUVC!).

Lepanthes elizabethae is similar to *L. lycocephala*, from which it is easily distinguished by the minute plant habit, triangular, strongly acuminate upper lobe of the petals, dolabriform blades of the lip without erect lobes near the base, and rounded, trilobed appendix of the lip.

Epiphytic, minute *plant*, up to 13 mm tall. *Roots* filiform, 0.5 mm in diameter. *Ramicaul* slender, erect, ca. 3.7 mm, covered by 2–4 ribbed lepanthiform sheaths with the ribs minutely denticulate and ostia minutely ciliate. *Leaves* suborbicular to obovate, obtuse, the apex slightly folded towards the abaxial surface, the base cuneate, contracted into a petiole 0.8 mm long, margin slightly undulate, shortly ciliate, 7.4 × ca. 5.0 mm. *Inflorescence* racemose, secund, successive, dense, 3.2 mm long, including the peduncle 1.6 mm long, borne from the abaxial surface of the leaf. Floral bracts cylindrical, 0.3–0.5 mm long. *Pedicel* 1 mm; *ovary* 1.2 mm long, smooth. *Flowers* minute, pink-reddish; slightly stained with yellow at the base of the sepals and petals. *Sepals* membranaceous, glabrous, ovate, acute, mucronate; the dorsal one triveined, entire, 3.6 × 2.1 mm,

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including a tail 1 mm long; the lateral ones connate to 1.8 mm, biveined, margin strongly dentate, teeth obtuse, 5.3 × 2.0 mm, including a tail 2 mm long. *Petals* minutely pubescent, transversely bilobed, 1.0 × 5.4 mm; the upper lobe triangular, strongly acuminate, 2.4 mm long; the lower lobe triangular, ending in a tail, 3.0 mm long, excluding the tail 2.1 mm long. **Lip bilaminate,** basally adnate to the column, pubescent, blades dolabriform, base of the blades obtuse, apex truncate, connective cuneate, 1.3 × ca. 2.0 mm, the appendix small, rounded, tomentose, parallel to the connective, trilobed, the lobes short, obtuse. *Column* arcuate, 1.7 mm long spread, with a pair of rounded apical wings. *Pollinia* 2, pyriform, ca. 0.5 mm long. *Anther cap* cordate in outline, base truncate, cucullate, translucent, 2-celled.

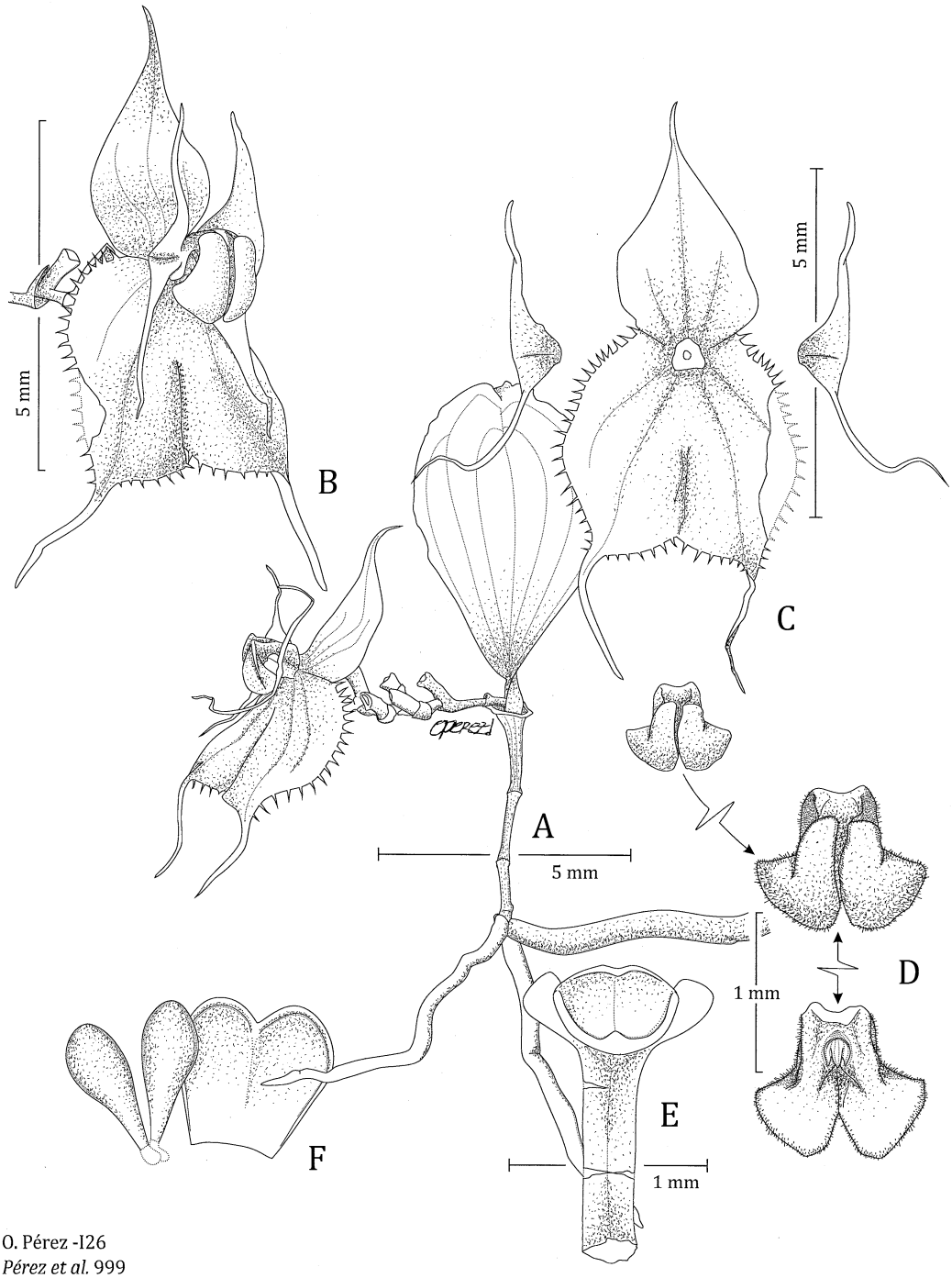

FIGURE 1. Illustration of *Lepanthes elizabethae*. A. Plant habit. B. Flower. C. Floral dissection. D. Lip details. E. Column. F. Pollinarium and anther cap; drawn by O. Pérez from the holotype.

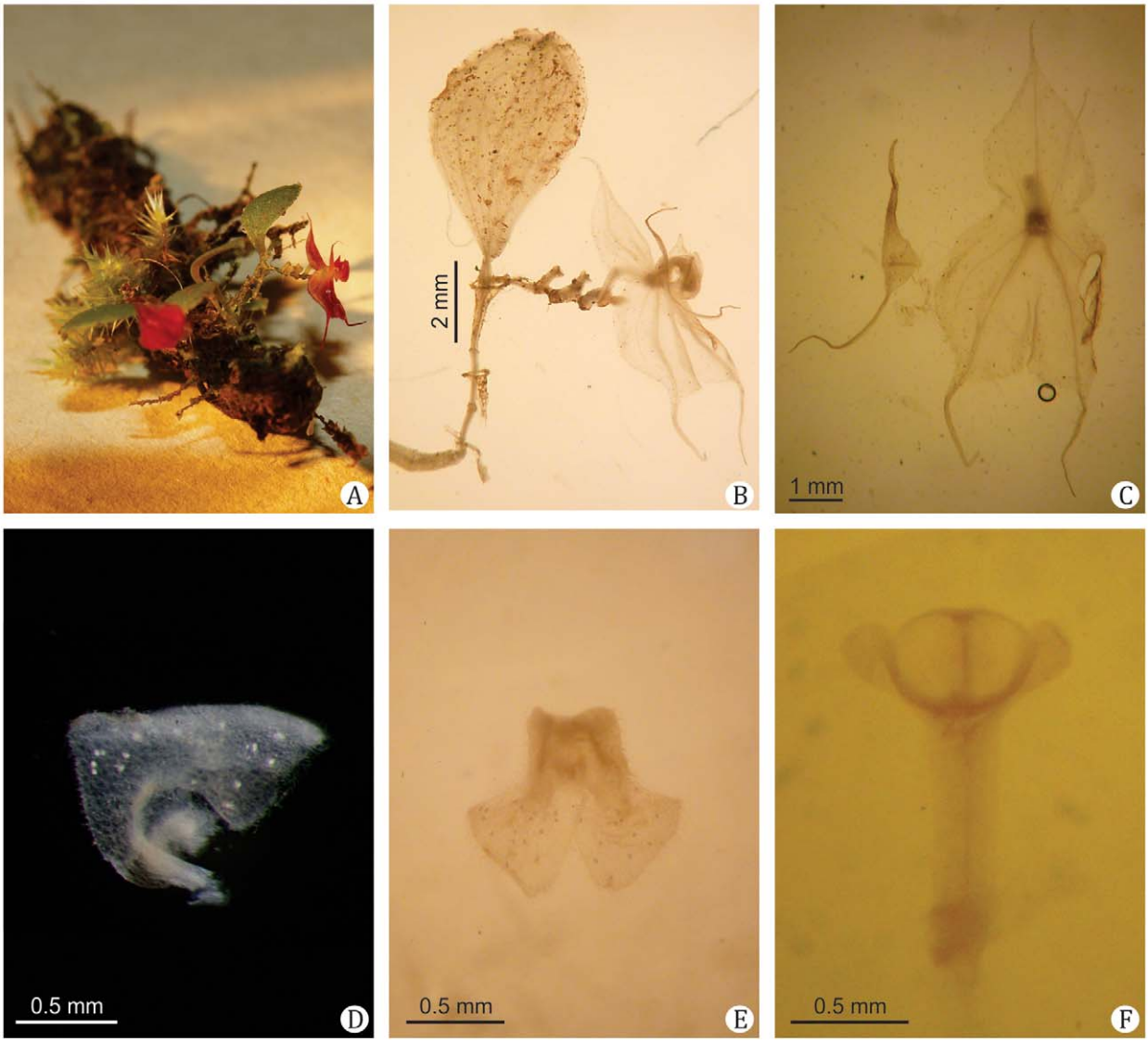

FIGURE 2. *Lepanthes elizabethae*. A. Habit. B. Flowering plant. C. Sepals and one petal. D. Lip (side view). E. Lip (ventral view—note the appendix just below the sinus). F. Column. (Photos O. Pérez.)

Distribution and habitat:—*Lepanthes elizabethae* is only known from the eastern slope of the Western Cordillera of the Andes, vicinity of Dapa, department of Valle del Cauca, Colombia (figure 3). It grows epiphytically in remnant cloud forest at about 1800–2000 m elevation. Plants were found growing on *Tibouchina sp.* (Melastomataceae) inside the forest, near creeks.

Conservation status:—According to the IUCN Red List (IUCN 2011), the species can be assigned as critically endangered (CR, criterion D2–very small or restricted population) due to the small population found only in the vicinity of Dapa in Colombia.

Eponymy:—Named after Elizabeth Santiago Ayala, researcher at the AMO herbarium, who has greatly contributed to the taxonomy of *Epidendrum*.

Discussion:—*Lepanthes elizabethae* is closely related to *L. lycocephala* Luer & Escobar (1984: 147), from which it differs by its minute habit, less than 1.3 cm tall, subrounded to obovate leaves (*vs.* ovate to elliptical, acute to subacute), length of the sepalar tails (2 mm long in *L. elizabethae vs.* 1 mm in *L. lycocephala*), petals with the upper lobe triangular, strongly acuminate (*vs.* triangular, narrowly obtuse), blades of the lip dolabriform with an obtuse base (*vs.* zoomorphic lip, blades subquadrate with acute lobes at the base) and rounded, tomentose lip appendix (*vs.* oblong, pubescent). All differences between these species are summarized in the Table 1.

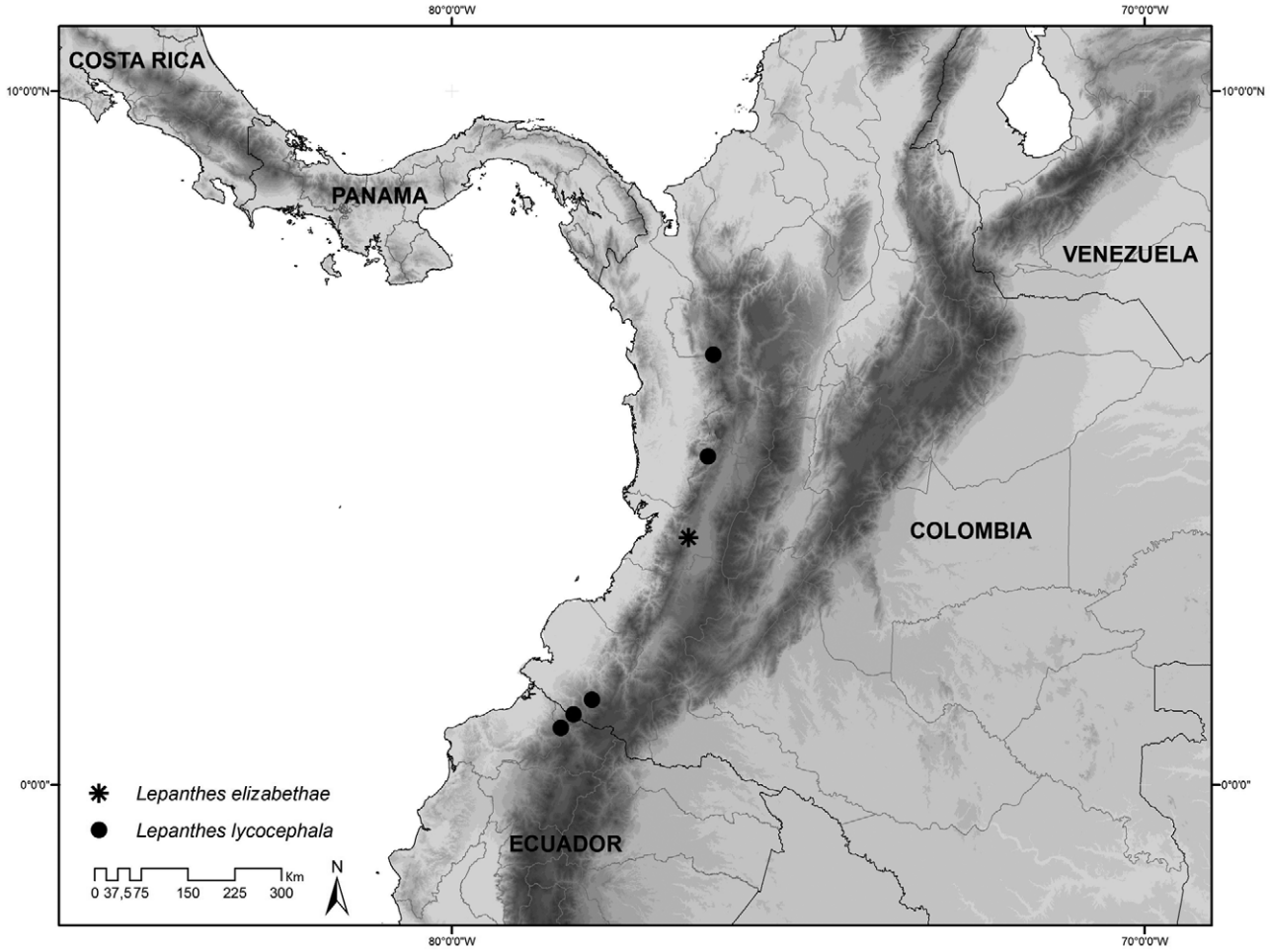

FIGURE 3. Distribution of *Lepanthes lycocephala* (based on the herbarium specimens *Luer et al. 4626* SEL!, *Luer et al. 15554, 16815, 17649* MO, *Luer 16815* MO, and *Hirtz 5866* MO) and *L. elizabethae* (type locality).

TABLE 1. Main differences between *L. elizabethae* and *L. lycocephala.*

	Lepanthes elizabethae	*Lepanthes lycocephala*
Plant size	up to 13 mm	up to 70 mm
Leaves	subrounded to obovate 7.4 × ca. 5.0 mm	ovate to elliptical 7.0–18.0 × 5.0–6.0 mm
Sepals	margin entire (dorsal sepal); tails up to 2 mm long	margin minutely denticulate (dorsal sepal); tails 1 mm long
Petals	1.00 × 5.30 mm; upper lobe strongly acuminate	0.75–1.00 × 3.75–4.00 mm; upper lobe narrowly obtuse
Lip	base of the blades obtuse	with erect, acute lobes near the base
Lip appendix	rounded, trilobed; the lobes short, obtuse, tomentose	oblong, pubescent (Luer 1984), or with a massive tuft of long hairs (Luer 1996).

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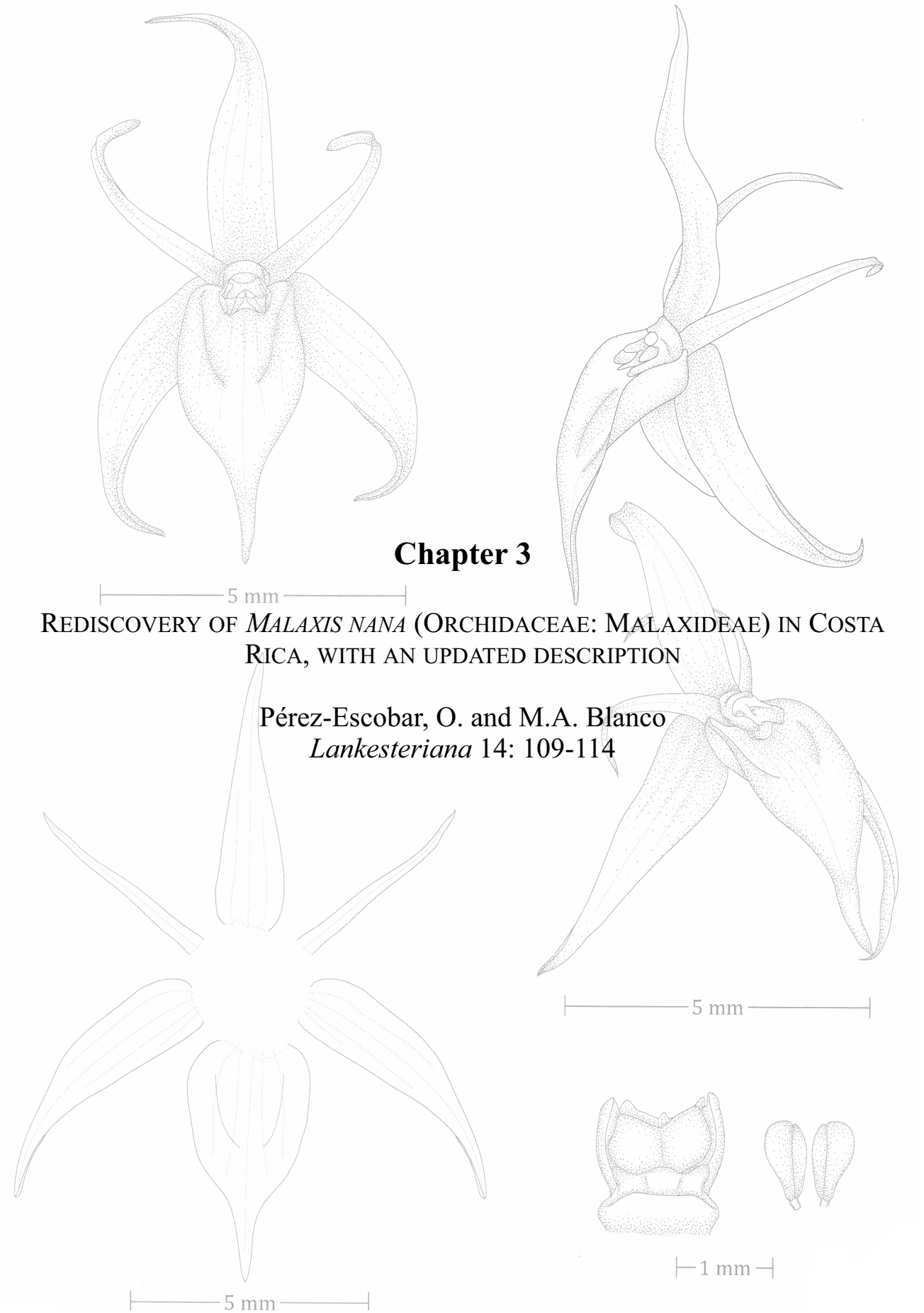

Chapter 3

Rediscovery of *Malaxis nana* (Orchidaceae: Malaxideae) in Costa Rica, with an updated description

Pérez-Escobar, O. and M.A. Blanco
Lankesteriana 14: 109-114

LANKESTERIANA 14(2): 109—114. 2014.

REDISCOVERY OF *MALAXIS NANA* (ORCHIDACEAE: MALAXIDEAE) IN COSTA RICA, WITH AN UPDATED DESCRIPTION

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Abstract. *Malaxis nana* C. Schweinf. is known from two herbarium specimens collected in 1925 in San Ramón, Alajuela province, and three additional specimens without detailed locality data collected in the late 1800's, all of them in Costa Rica. This species had not been registered since. *Malaxis nana* is hereby first reported for Las Cruces Biological Station, Puntarenas province, in southern Costa Rica. An updated description, illustration, photographs and distribution map for this taxon are provided.

Resumen. *Malaxis nana* C. Schweinf. se conoce de dos especímenes recolectados en 1925 en San Ramón, provincia de Alajuela, y tres especímenes adicionales sin datos de localidad detallados y recolectados en los finales de los 1800's, todos de Costa Rica. Esta especie no había sido registrada desde entonces. Aquí informamos por vez primera sobre la existencia de *Malaxis nana* en la Estación Biológica Las Cruces, provincia de Puntarenas, en el sur de Costa Rica. Se presenta una descripción actualizada, ilustración, fotografías y mapa de distribución para este taxón.

Key words: Alberto M. Brenes, Auguste R. Endrés, Las Cruces Biological Station

Introduction. The genus *Malaxis* Sol. ex Sw. (1788: 119; Orchidaceae) encompasses ca. 300 species (Todzia 1995, Dodson 2002, Dressler 2003, Cribb 2005) distributed worldwide, with at ca. 100 species in the Western Hemisphere (Dodson 2002) and 21 reported so far for Costa Rica (Pupulin 2002, Dressler 2003). According to a preliminary molecular phylogenetic analysis (Cameron 2005) the genus is at least diphyletic in its traditional circumscription. Here, we adopt the generic classification of tribe Malaxideae Lindl. of Cribb (2005; 13 genera), as well as his circumscription of *Malaxis*. Szlachetko and Margońska (2006) recognize at least two generic segregates of Neotropical *Malaxis* sensu Cribb (2005) (i.e., *Microstylis* (Nutt.) Eaton and *Tamayorkis* Szlach.); however, their rationale is not explicit, and the species treated here would still be included in their narrow circumscription of *Malaxis*.

Tropical species of *Malaxis* occur in a great variety of environments, ranging from lowlands rain forests to paramos (and reportedly also from semiarid environments; González-Tamayo 2002), from sea level to 3500 m elevation (González-Tamayo 2002). Plants of *Malaxis* are easily recognized by their herbaceous, sympodial habit, rhizomatous stems often with small pseudobulbs or corms covered by membranaceous cataphylls, one or two non-articulated leaves produced per sympodial unit, terminal inflorescences (either racemes or corymbs), and small, usually green flowers with a frequently concave disc (sometimes transversally divided by a longitudinal ridge) located at the base of the labellum.

During the botanical field course "Sistemática de Plantas Tropicales (OET 2013-18)" at Las Cruces Biological Station (southern Fila Costeña, Puntarenas Province, Costa Rica), a small epiphytic plant of *Malaxis* was found growing in late secondary forest at the base of a mature tree with ca. 50 cm of diameter at breast height (DBH); this plant was eventually identified as *M. nana* C. Schweinf. (1938: 89–91).

After studying specimens from six herbaria in Costa Rica (CR, HLDG, INB, JBL, LSCR, and USJ) and other important herbarium databases available on-line (AMES, K, MO, NY and W), only three additional specimens of *M. nana* were found (*Endres 138* and *Endres s.n.* [2 specimens], both at W, collected somewhere in Costa Rica between 1866 and 1874; see discussion).

Because of the dearth of information on *Malaxis nana*, we provide an updated description, illustrations, a distribution map, and brief commentaries on the ecology of this taxon.

Materials and methods. Live plants of *Malaxis nana* were collected on July 2013 in the forest preserve of Las Cruces Biological Station (see detailed locality data under "additional specimens examined", below). The identification was made using the treatment of Dressler (2003) and verified by comparing the plant with the protologue (Schweinfurth 1938). A dry herbarium specimen was prepared, and flowers were also preserved in liquid (70% ethanol, 20% water, 10% glycerol). The updated description below was prepared based on all six collections of *M. nana* available to us (either as physical specimens or as digital images) by early 2014. Distribution maps were generated using DIVA-GIS.

Taxonomic treatment

Malaxis nana C. Schweinf., Bot. Mus. Leafl. 5(6): 89–91. 1938. (Figs. 1, 2)

Type: —COSTA RICA. [Alajuela: San Ramón,] bois à San Pedro de San Ramón, epiphyte, de 7 cm. haut., alt. 850 m, 27 June 1925, *Brenes* (*96*) *1301* (holotype: AMES [image!], mounted on same sheet as paratype).

Epiphytic, sympodial, cespitose *herbs* (usually with only 2 consecutive sympodial units present at any given moment), 2–6 cm tall (to the top of the inflorescence). *Roots* 1.0–1.9 mm in diameter, whitish, pilose, growing from the base of each pseudobulb. *Pseudobulbs* 5–13 × 4–6 mm, green, ellipsoid to ovoid, heteroblastic, covered by 1–2 membranaceous cataphylls 0.5–2.0 cm long. *Leaves* 2 per sympodial unit (produced from the apex of the pseudobulb), present only in the most recent sympodial unit, shortly pseudopetiolate; pseudopetioles (sheaths of the foliage leaves) U-shaped in cross section, 5–27 × 3–4 mm (folded), erect, enveloping each other and the inflorescence, forming a pseudostem that projects above the hidden pseudobulb; blades 13.0−68.0 × 2.1–36.0 mm (in flowering shoots), often slightly anisophyllous, horizontal to ascending, subopposite, broadly lanceolate to ovate, basally cuneate to round, apically acute, shiny green with crystalline texture adaxially, matte greyish green abaxially, herbaceous, 9–16 veined, the midvein impressed. *Inflorescences* 22–45 mm long (including peduncle), erect to arcuate; peduncle 23–41 mm long, minutely ribbed, of a single visible internode; rachis 2–4 mm long, corymbose, with up to 25 simultaneously open flowers and ca. 12 developing buds. *Floral bracts* up to 2 × 1 mm, spreading, membranaceous, green, triangular, 1-veined. *Pedicel* plus *ovary* 5–15 mm long, seemingly increasing in length with age during both before and during anthesis. *Flowers* relatively big for the size of the plant (open perianth ca. 9 mm long), secondarily non-resupinate (by 180 degree twisting and upward bending of the pedicel), emerald green, turning coppery orange when old (or "chestnut brown" according to one herbarium collection), membranaceous, sepals and petals lustrous and somewhat translucent. *Dorsal sepal* 6.0–7.6 × 1.2–2.0 mm, spreading, adpressed to the ovary, narrowly lanceolate, acute to long-acuminate, entire, retrorse towards the apex, 3-veined. *Lateral sepals* 6.1–8.0 × 1.8 mm, free, spreading, obliquely narrowly lanceolate, acute to acuminate, entire, margins slightly revolute, 3-veined. *Petals* 6.0–7.0 × 0.5–1.0 mm, spreading, slightly recurved upon the middle part, narrowly triangular to linear, acute, entire, 1-veined. *Labellum* 5.1–6.5 × 2.1–4.0 mm, spreading, ovate to lanceolate, long-acuminate, entire, fleshy, concave at the proximal half, disc cavity non-divided, somewhat darker than the rest of the labellum. *Column* 1.1 × 2.0 mm (wider than long), dorsiventrally compressed, emarginate. *Anther* dorsal, with 2 divergent thecae. *Pollinia* 4 in 2 hemipollinaria (1 per theca), each pollinium ca. 1 mm long, yellow, narrowly ovoid; the two pollinia in each hemipollinarium tightly appressed to each other, sharing a single apical caudicle ca. 0.2 mm long. *Rostellum* concave. *Stigma* ventral, transversally bilobed, ca. 0.7 × 1.5 mm. *Fruit* a capsule, 5 mm long when dehisced, apparently with 2 narrow valves and 1 wider valve separating at apex.

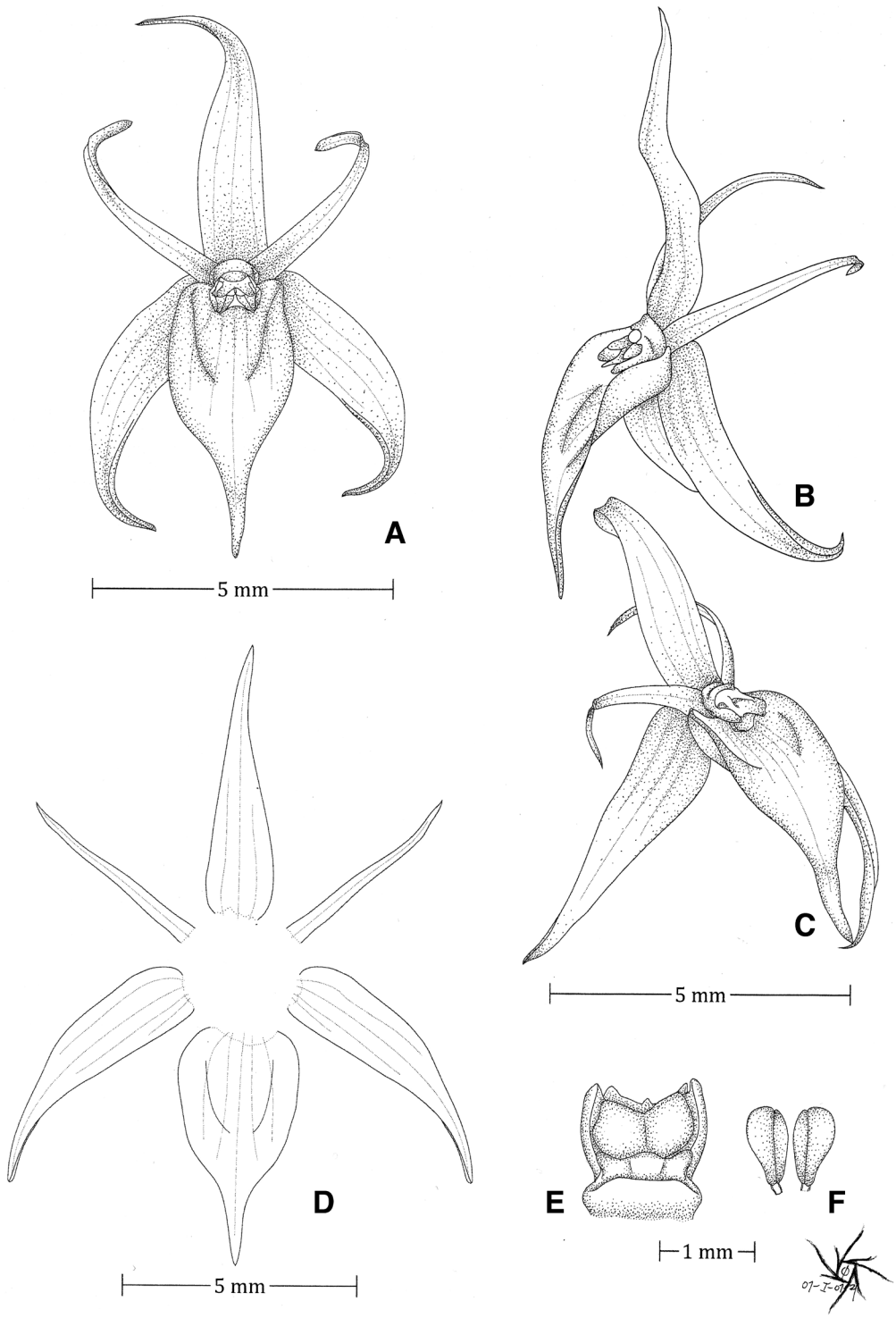

Figure 1. *Malaxis nana*. A. Flower, front view. B. Flower, side view. C. Flower, oblique view. D. Dissected perianth. E. Column, dorsal view, with hemipollinaria removed. F. Hemipollinaria. Drawn by O. Pérez from *Pérez 1412*.

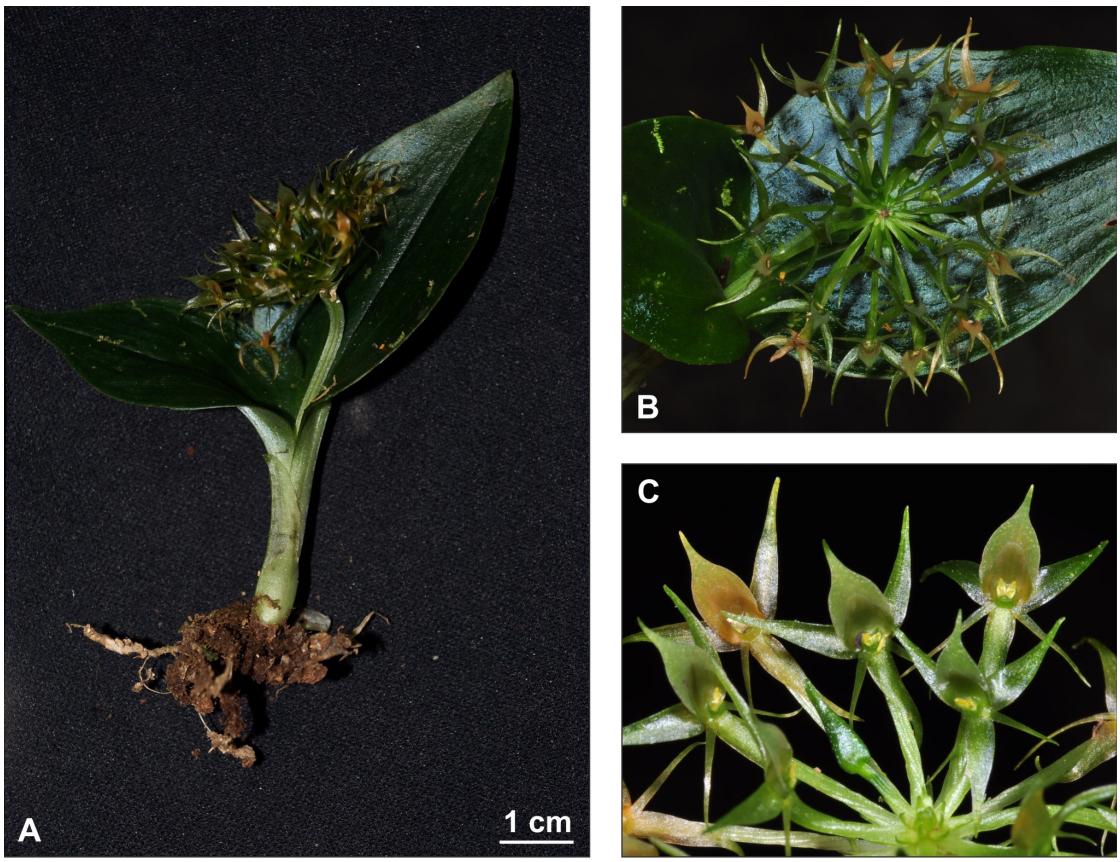

Figure 2. *Malaxis nana*. A. Plant habit. B. Inflorescence, top view. C. Flowers, top view. Note flowers secondarily non-resupinate by the 180 degree twisting and upward bending of the pedicels, and the old flowers turning yellowish orange. Photos by M. A. Blanco & O. Pérez.

Additional specimens examined: COSTA RICA. Without additional data: *Endrés s.n.* (W no. 1889-39091, image!), 1867, *Endrés 138* (W no. 19521 [image!] & 1889-40326 [image!]). [Alajuela: San Ramón,] Bosquet du Cerro de San Isidro de San Ramón, 1175 m, 10 July 1925, *Brenes (131) 1334* (AMES [image!, mounted on same sheet as holotype], CR!). Puntarenas: Coto Brus, San Vito, Estación Biológica Las Cruces, sendero Río Java, 1200 m, lat.: 8.786788°, long.: -82.965540°, 14 July 2013, *Pérez 1412* (USJ!, JBL-liquid!).

Distribution and ecology: *Malaxis nana* is considered endemic to Costa Rica and so far it is known only from Alajuela Province, San Ramón County (type locality and San Isidro Hill) and Puntarenas Province, Coto Brus County (Las Cruces Biological Station) (Fig. 3). The last locality is only 6 km away from the Panamanian border; thus, it is highly likely that the species also occurs in Panama.

Plants of *Malaxis nana* grow as epiphytes in the lower strata of premontane wet forests, in an elevational range of 850–1200 m. In Las Cruces Biological Station, plants of *M. nana* were observed growing on mature trees of ca. 50 cm DBH. When the present manuscript was in press, we learned that *M. nana* was collected again in Las Cruces Biological Station in June 2014, this time during the course "Tropical Plant Systematics" (voucher: *Bonifacino & Damián 5001*, to be deposited at USJ; verified by photos of the live plant sent to us), apparently from the very same colony as *Pérez 1412*. Flowering plants have been collected at least in June and July (the Endrés specimens do not indicate a collecting date).

Commentary: *Malaxis nana* was described by Charles Schweinfurth (1938: 89–91) from a plant collected

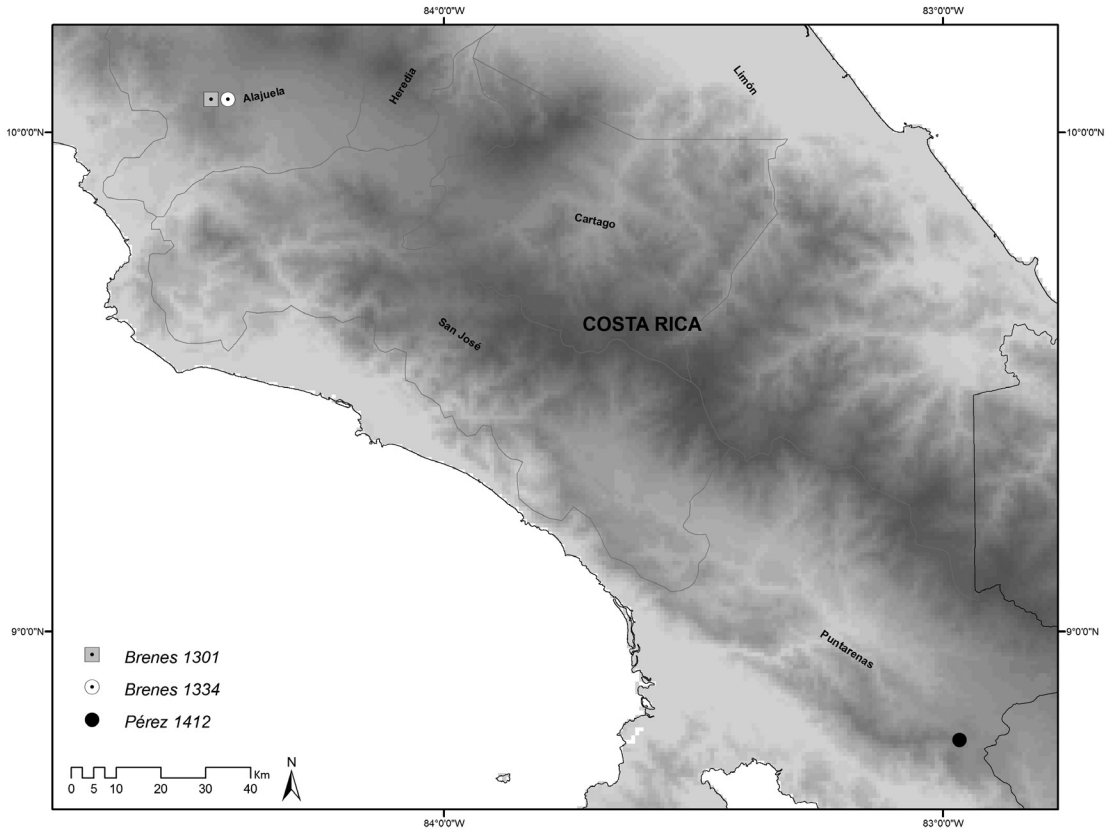

Figure 3. Distribution of *Malaxis nana* (based on available herbarium specimens with locality data)

in San Ramón county, Alajuela province, and from another record from a nearby locality (San Isidro Hill); both plants were collected by Alberto M. Brenes (for information about Brenes's collecting activities and the numbering of his collections see Barringer 1986).

Three other herbarium specimens (unknown to Schweinfurth) were collected by Auguste R. Endrés in Costa Rica sometime between 1866 and 1874 (during his stay in that country; Ossenbach *et al*. 2010) – at least two of them in 1867 (see below). Endrés sent his specimens to H.G. Reichenbach in Hamburg, and they are currently deposited in the herbarium of the Naturhistorische Museum in Vienna (W). These three specimens lack additional locality information; however, they were possibly collected in the region of San Ramón, where Endrés lived during most of his time in Costa Rica (Ossenbach *et al*. 2010). Like many other collections that Endrés sent to Reichenbach, these represented a then-undescribed species but Reichenbach never described it (see Pupulin *et al*. 2011). They were identified as *Malaxis nana* by Robert L. Dressler in 2001. Images of these and other Endrés collections are available through the Virtual Herbaria website (http://herbarium.univie.ac.at/database/search.php).

Two of the Endrés specimens have attached pieces of rag paper with the handwritten annotation "1867 […] Nº 138 *Microstylis* − fls. chestnutbrown (concolored)". The first number probably refers to the year of collection. The second number is the "species number"; Endrés did not use collection numbers in the modern sense (i.e., to designate gatherings), but he used these numbers to correlate drawings and descriptions with plants that in his opinion belonged to the same species (Pupulin *et al*. 2011). It is interesting that the flowers of these specimens were described as "chestnut brown", in contrast to the plant from Las Cruces, which had green flowers. None of the two Brenes collections have a description of the flower color.

In spite of more or less constant and intensive botanical explorations in Costa Rica ever since, no additional collections of this species were known until now. After 73 years of its description, hereby another population is reported, growing on a premontane wet forest from Puntarenas province, Coto Brus County (ca. 220 km SE from the type locality). *Malaxis nana* can be recognized from other Costa Rican congeners by the small size of the plant, very short pseudobulbs bearing two leaves each, very short rhizome segments, thyrsoid inflorescences, relatively large flowers, the entire, acuminate labellum without auricles or lobes at the base, and the non divided disc cavity.

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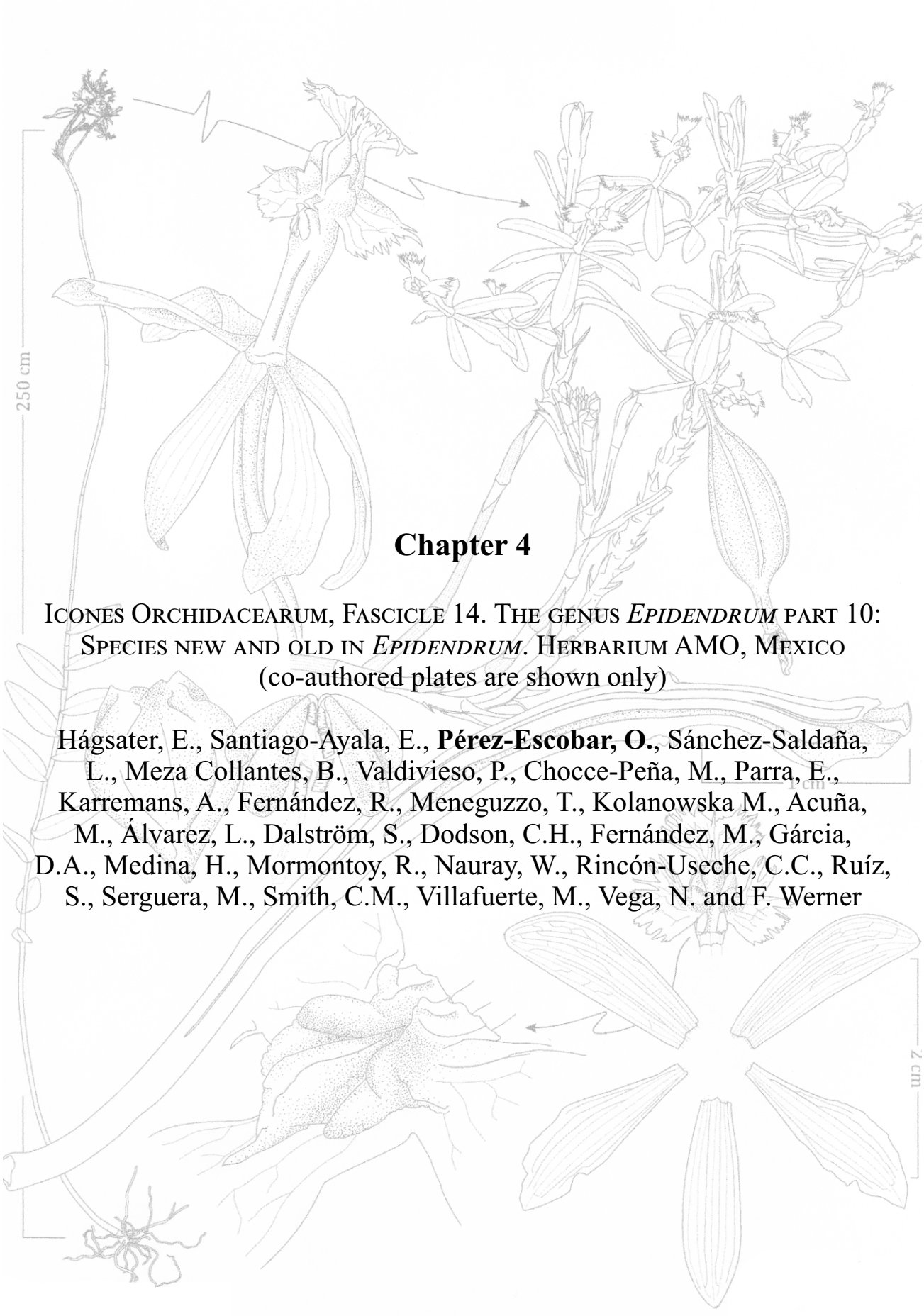

Chapter 4

Icones Orchidacearum, Fascicle 14. The genus *Epidendrum* part 10: Species new and old in *Epidendrum*. Herbarium AMO, Mexico
(co-authored plates are shown only)

Hágsater, E., Santiago-Ayala, E., **Pérez-Escobar, O.**, Sánchez-Saldaña, L., Meza Collantes, B., Valdivieso, P., Chocce-Peña, M., Parra, E., Karremans, A., Fernández, R., Meneguzzo, T., Kolanowska M., Acuña, M., Álvarez, L., Dalström, S., Dodson, C.H., Fernández, M., Gárcia, D.A., Medina, H., Mormontoy, R., Nauray, W., Rincón-Useche, C.C., Ruíz, S., Serguera, M., Smith, C.M., Villafuerte, M., Vega, N. and F. Werner

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ICONES ORCHIDACEARUM

Fascicle 14
THE GENUS EPIDENDRUM
Part 10
"Species New & Old in Epidendrum"

In memoriam Pedro Ortiz Valdivieso, S.J.

Eric Hágsater

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ICONES ORCHIDACEARUM

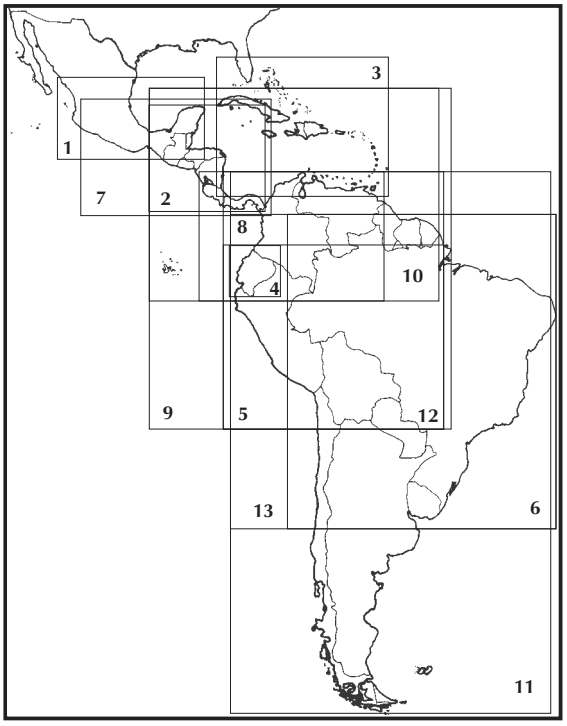
Fascicle 14, plates 1401 to 1500

THE GENUS EPIDENDRUM
Part 10

"Species New & Old in Epidendrum"

Reference Map

TROPICAL AMERICA
(numbers refer to the portions of the map used in individual plates)



ICONES ORCHIDACEARUM

Fascicle 14, plates 1401 to 1500

THE GENUS *EPIDENDRUM*
Part 10
Species New & Old in *Epidendrum*
In memoriam Pedro Ortiz Valdivieso, S.J.

Foreword

We dedicate this volume to the memory of Father Pedro Ortiz Valdivieso, S.J., (31 January 1926 – 18 July 2012) who passed away last year in Bogota. He was an inspiration not only for Colombian orchidists, but for all who met him, and had the pleasure of visiting him or going out into the field with him. We had been corresponding since the early 70's; he shared his material with us, and we herewith dedicate a new species to him. He also co-authors two new species, shared his photographic material, and acted as a reviewer for several texts. In addition, we appreciate the Latin translation of the diagnoses, when that was still a requirement in the Botanical Code of Nomenclature.

In this volume we present three novelties. First all texts have been reviewed by at least two reviewers, a process which has definitely helped in catching mistakes, sometimes questioning the author´s information or pointing out additional information which had not been originally considered. Second, we have added, where possible colored images of the species described. Third, the printed edition is published simultaneously with the electronic copy, which are identical. The electronic version is freely available to all at http://www.herbarioamo.org/index_archivos/Fascicle14.pdf; whereas the printed copy is sent to libraries, as well as the subscribers and authors. The electronic texts are in searchable pdf form. The participation of 28 authors and co-authors, 12 illustrators, and 43 photographers is appreciated, as well as 32 reviewers.

Up to now we have used the abbreviation used by Tropicos of the Missouri Botanical Garden, **Icon. Orchid. (Mexico)**. However, the IPNI International Plant Nomenclature Index, a consortium including the Royal Botanic Gardens, Kew, and the Harvard University Herbaria indicate the abbreviation simply as **Icon. Orchid.** which we herewith adopt.

We herewith present 77 species new to science, distributed from Mexico, through Central and South America, as far as Argentina. They are distributed in Colombia (33), Peru (27), Ecuador (15), Costa Rica (5), Brazil (3), French Guiana (2), and one each from Argentina, Bolivia, Mexico, Panama, Paraguay, Surinam, and Venezuela. They do not add up because some species are reported from more than one country.

Much material from Colombia has been studied, in preparation for the Orchids of the Valle del Cauca, which will be published shortly by Dariusz Szlachetko *et al.*, where the team from the Herbario AMO has collaborated in the preparation of the genus *Epidendrum*. Much information has been provided by our Colombian collaborators, especially Oscar Alejandro Pérez Escobar and Edicson Parra Sánchez, but also many others.

We continue to work closely with several Peruvian botanists who have co-authored numerous species of that country, especially Benjamín Collantes Meza. We have been surprised to find the diversity between the northern, central and southern parts of Peru. Most of the older collections were made in the north, so having access to material from throughout the country, interesting differences appear. We illustrate the true *Epidendrum paniculatum* Ruiz and Pav. after piecing together the type material found in Madrid, and thanks to the help and information provided in part by Franco Pupulin and the curators of MA which we recently visited.

In tackling the Paniculatum Sub-group, we also took on the Brazilian species which have often been confused with that species, but represent a different group, the Densiflorum Group. Most specimens had been identified as *Epidendrum densiflorum* Hook., but aside from the new *Epidendrum andres-johnsonii* Hágsater & E.Santiago, we also recognize *E. brachythrysus* Kraenzl., *E. hassleri* Cogn, *E. lindbergii* Rchb.f., and *E. noackii* Cogn., all rather widespread in the southern half of Brazil and most down around Foz do Iguaçu, including neighboring Argentina and Paraguay. We wish to thank various Brazilian and Argentine amateurs and botanists for their information, and in particular Thiago E. C. Meneguzzo for his critical revision of this group. We wish to thank the curator of the herbaria CTES María Mercedes Arbo, as well as Irma Stella Insaurralde, Enrique Gandolla, and Miriam Valebella, all in Argentina.

A new sub-group within the Pseudepidendrum Group is established: the new Pluriracemosum Sub-group, which though similar to the Paniculatum Sub-group, it is recognized by the fact that it produces new racemes from the old inflorescence. The flowers are somewhat variable in color, from the basic green and white to purple-brown and pink and yellow. *Epidendrum unguiculatum* (C.Schweinf.) Garay & Dunst. and *E. iguagoi* Hágsater & Dodson belong here, together with the new *E. pluriracemosum* Hágsater & E.Santiago, *E. humantupanorum* Hágsater & E.Santiago, and *E. oenochrochilum* Hágsater, Ric.Fernández & E.Santiago.

Finally, *Epidendrum hemihenomenum* Hágsater & Dodson is illustrated from new material from Peru. The original drawing and description had been prepared from a poor flower in alcohol and photographs from Ecuador. This adds and corrects various details. Other corrections and additions to previously published icons are found in the appendix.

Eric Hágsater

Mexico City, May 2013.

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"I found the gene of love in the orchids, the same that must awaken the heart in human beings"

PEDRO ORTIZ VALDIVIESO S.J.
January 31, 1926 – July 18, 2012

Germán Ortiz Plata*

We are fortunate to have known and shared moments in our lives with Father Pedro Ortiz, a special person who taught us to appreciate the marvels of creation and with his life showed us what we can achieve with faith and perseverance.

Since an early age, he left his native Santander to pursue the Jesuit road, initially in the United States and later in various European countries. He was ordained as priest in Austria, and later studied a Doctorate in Sacred Scriptures in the Pontifical Institute in Rome.

As translator of the Bible and facilitator of critical instruments for its study, he was part of a team that produced the latest Spanish version edited by the United Biblical Societies, entitled "Dios habla hoy". In churches across Colombia today, every Sunday you listen to the Gospels of the New Testament which were translated into a version adapted to the popular language of this country. Many Colombian priests were his alumni in the Faculty of Theology at the Pontifical Xavierian University, of which he was a professor and dean for many years.

His studies lead him to learn over 10 languages and several dead ones, tools necessary for his professional work. Maybe that is why his discourse was characterized by its precision and effectiveness. He did not dedicate his time to banalities, and his search for the reason behind things was rigorously scientific. In addition to his outstanding work, he liked sciences and art, such as painting, photography, music and writing, astronomy, technology and botany. His taste for science lead him to study orchids. He enjoyed nature, esthetical taste and had scientific curiosity, which were all joined in the beauty of orchids which captivated him for the rest of his life.

He explored mountains and books like a scholar, photographed and illustrated like an artist, and studied and classified orchids with the patience of a researcher, and taught us with generosity and dedication. In spite of his deep knowledge of Colombian orchids, he never considered any plant as his own; the fact leaves us food for thought. His answers to the most simple or complicated query, made by friends and strangers alike, were never late in arriving. His legate to orchidology, more than his teachings in direct conversation, correspondence or lectures has been compiled in numerous books and articles, where he authored 105 taxa, covering new genera, species, varieties and combinations.

In spite of his saying he was no more than an amateur orchidophile, during his last years he worked on the Orchid Molecular Botanical Expedition project, supported by the Pontificia Universidad Javeriana. His objective in this field was the evaluation of genetic variability in Colombian orchids, extracting several DNA molecular markers.

He has left us his work, enthusiasm, his dedication and commitment, detachment, and love for people and things done, reminding us of the phrase that as a follower of St. Ignatius of Loyola guided his life *"Know, love, serve Christ and be happy with God forever"*.

References: Without author, 2012, Bibliographia Orchidologica Ortiziana, **Lankesteriana** 12(2): 84-92. Ortiz Plata, Germán, 2012, Pedro Ortiz Valdivieso, **Orquideología** XXIX(2): 141-142.

Photographic credit: We were unable to determine the photographer, the digital image was found on Father Ortiz´s computer after he passed away. This article is translated from the original cited above; published with permission of the author and the editor.

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ICONES ORCHIDACEARUM

Fascicle 14, plates 1401 to 1500

THE GENUS *EPIDENDRUM*
Part 10

"Species New* & Old in *Epidendrum*"

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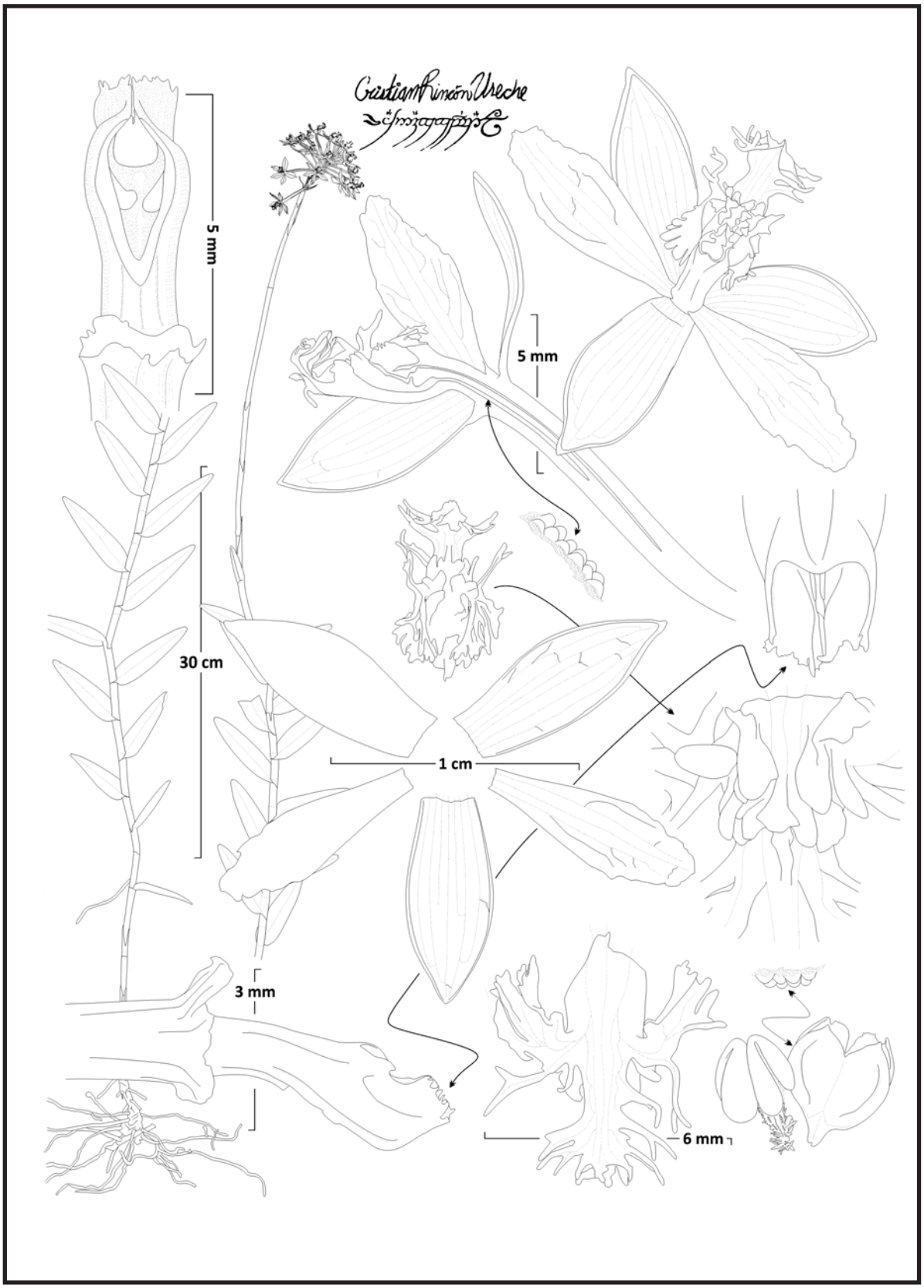


EPIDENDRUM AURA-USECHEAE Hagsatér, C.Rincón-Useche & O.Pérez **Plate 1410**

EPIDENDRUM AURA-USECHEAE Hágsater, C.Rincón-Useche *et* O.Pérez, *sp. nov.*

Type: COLOMBIA: Cundinamarca: Municipio de Junín; Vereda San Rafael, ca.1600 m, ca. 23° C, 20 Abril 2011, ***Cristian Camilo Rincón Useche 003.*** Holotype: COL! (Illustration voucher). Isotype: CUVC!

Simile *Epidendri xanthini* Lindl. sed margine apicali petalorum undulato, callo acervato labelli composito 12-14 tuberculis inaequalibus et margine labelli profunde fimbriato, fimbriis geniculatis quaquaversum fingentibus plane absentiam ordinis.

Terrestrial or lithophytic, sympodial, cespitose, erect **herb**, 54-142 cm tall including the inflorescence**. Roots** fleshy, 0.7-2.1 mm, basal, thick. **Stems** 38-105 x 0.35-0.86 cm, simple, cane-like, erect when young, arching when mature, purple at the base, pale purple in the middle, apically green. **Leaves** 26 distichous, alternate, dark green, distributed throughout upper 2/3 of the stem; sheaths 19.6-34.8 x 3.7-8.6 mm, tubular, striated; blade 3-12 x 0.7-2.0 cm, narrowly elliptic-lanceolate, apex obtuse, faintly bilobed, coriaceous, smooth, green, unequal in size, the lower and upper leaves smaller. **Spathaceous bract** lacking. **Inflorescence** apical, racemose, successive, pluri-racemose (producing new racemes through the time); each raceme compact, many-flowered, dense, peduncle elongate, terete up to 42.5 cm long; covered by several tubular, acute, imbricating bracts, 4.2-7 cm long. **Flowers** numerous, successive, 12-16 open at one time, non-resupinate, yellow, callus yellow to orange in mature flowers, column orange; no fragrance recorded. **Floral bracts** 3.1-12 mm long, much shorter than the ovary, triangular, acuminate. **Ovary** 3.27-3.95 cm long, thin, terete, not inflated. **Sepals** 8.3-9.2 x 3.2-3.8 mm, spreading, free, elliptic, slightly acute at the apex, 6-7-veined, margin entire; the **lateral** sepals with a low raised, dorsal keel. **Petals** 9 x 3.2 mm, free, spreading, obovate-spatulate, obtuse, margin erose along the apical half, basal half entire, 3-veined, lateral veins branching from the middle. **Lip** 7.5-8 x 6.5-7 mm spreading, united to the column, 3-lobed, base deeply cordate, margin deeply fimbriate, in natural position the fimbria are geniculate in all directions, appearing in total disorder; the calli complex, massive, occupying the isthmus and base of the lip, represented by a structure of 12-14 unequal tubercles, the basal pair and lateral pair more prominent; disc without keels; lateral lobes 2 x 4.2 mm, trapezoid, deeply emarginate towards the posterior margin, almost forming a pair of additional lobes, appearing to be 5, anterior margin folded horizontally; mid-lobe 3.3 x 3.4 mm, deltate, base forming an elongate isthmus, apex slightly folded toward the adaxial part of the lip. **Column** 4.0-4.7 mm long, short, slightly arched, thin, with a pair of prominent apical, upturned wings, the apical margin truncate, and irregularly dentate. **Clinandrium** reduced, margin entire. **Rostellum** apical, slit. **Lateral lobes of the stigmatic cavity** prominent, occupying 1/3 of the cavity. **Anther** obovoid, apiculate, papillose, 4-celled. **Pollinia** 4, obovoid, laterally compressed, sub-equal, caudicles longer than the pollinia, formed by tetrads which appear like a pile of roof tiles. **Nectary** penetrating 1/3 of the ovary, papillose. **Capsule** narrowly elliptic, pedicel 11-14 mm long, apical neck short.

OTHER SPECIMENS: COLOMBIA: Antioquia: Quebrada at head-waters of Río Tenche, near Carolina, 2080 m, 15 V 1944, *Core 719*, US! W slope of Cordillera Occidental, 50 km NW of Antioquia and 75 km SE of Uramita, 1810 m, 9 X 1977, *Gentry 20292*, COL! MO! SEL! Mun. Frontino, km 10 of road Nutibara-Murrí, 1970 m, 26 IX 1987, *Zarucchi 5814*, MO! **Boyacá:** entre Santamaría y Piedra-campana, 800-1100 m, 20-25 VII 1964, García-Barriga 18084, AMES! COL! Arcabuco-La Cumbre; ca. Serranía El Peligro, margen de carretera que conduce a Moniquirá, 2600 m, 02 IX 2011, *Pérez 1162,* VALLE! **Cundinamarca:** Alto de Quemara, Gazaunta Valley, 10 km NW of Medina, 1430 m, 5 X 1944, *Grant 10380*, COL! US! WIS! **Santander:** alrededores de Bucaramanga, 1500 m, 27 VIII 1948, *Araque 18S174*, AMES! MEDEL! US! along road to Tona, 3 km off Bucaramanga-Pamplona, 1950 m, 3 V 1983, *Croat 56401*, MO x2! Mpio. de Virolín, 1800 m, 6-12 V 1986, *Fernández Alonso 6203*, COL! (illustration, AMO!) between Piedecuesta and Las Vegas, 1200-2000 m, 19, XII 1926, *Killip 15473*, AMES! NY! US! Bucaramanga, ca. 1000, 16 II 1927, *Killip 19341*, AMES! NY! US! La Corcova (Tona), 1866 m, 12 X 1977, *Rentería 650 (6)*, COL! MO!

OTHER RECORDS: COLOMBIA: Digital images by Pedro Ortiz Valdivieso, published in Gallery of Colombian Orchids as *Epidendrum secundum* (*xanthinum*), CD,Bogotá, 2007. **Antioquia:** Guadalupe, *Camilo-Sánchez s.n.*, digital images by *Camilo-Sanchez*, AMO! Amalfi, *Vélez s.n.*, digital images, AMO! **Cundinamarca:** Tenjo, *Hurtado s.n.*, digital images by Ana B. Hurtado, AMO! **Valle del Cauca:** vía al mar km 13, 1400 m, 8 XI 2007, *J. Farfán s.n.*, digital image, AMO!

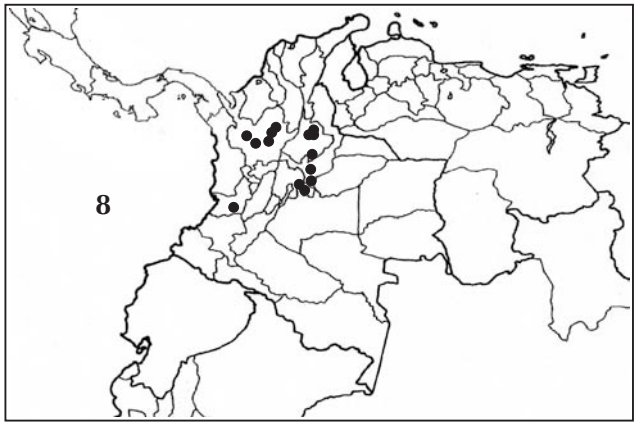
DISTRIBUTION AND ECOLOGY: Widespread in Colombia, registered north of Bogotá, in Antioquia, and Valle del Cauca in the south; in rocky outcrops in grassland and among low shrubs at 1200-2600 m altitude. Forming large populations and apparently not hybridizing (Farfán, pers. comm. 2011). Flowering in January to May.

RECOGNITION: *Epidendrum aura-usecheae* belongs to the Secundum Group which is recognized by the caespitose habit, numerous coriaceous leaves, and generally an elongate peduncle to a pluri-racemose inflorescence, brightly colored flowers generally pollinated by hummingbirds, and the caudicles of the pollinarium granulose, the tetrads appearing like a loose pile of roof-tiles, without any spathaceous bracts; and Elongatum Sub-group, recognized by the non-resupinate flowers with a complicated callus. This species is lithophytic with yellow flowers, the margins of the lip deeply fimbriate with the fimbria bent in all directions, giving an impression of total disorder; the column wings are prominent, bent upwards and apically truncate, the margin irregularly dentate. It is color-wise very similar to *Epidendrum xanthinum*, described from Minas Geraes, Brazil, which has the margins of the larger lip (7-8 x 9-10 mm), spreading flat, and deeply dentate. *Epidendrum melinanthum* Schltr. described from the Valle del Cauca has a much simpler callus formed by 3 tubercles, the lip is T shaped, with a deeply dentate margin, and the mid-lobe bifid, into two square, somewhat divergent lobes with a mucro in the sinus. The more common species in the area north of Bogotá is the purple-pink *Epidendrum arachnoglossum* Rchb.f. *Epidendrum fimbria* Rchb.f. has orange colored flowers, shorter leaves, ca. 3.5-7 cm long, the lateral lobes of the lip are semi-ovate with the margin lacerate, and the mid-lobe is cuneate with the dentate.

NOTE: It is curious that this entity appears to be widespread in Colombia. The shape and general disorder of the fimbria of the lip are easily visible feature, even in herbarium specimens, as are the generally narrow leaves.

ETYMOLOGY: Named in honor to Aura Delia Useche Barbosa, mother of the second author, who always has given him her knowledge and unconditional love and is his inspiration and moral support on field trips.

CONSERVATION STATUS: NT. Not threatened. Widespread and common in northern Colombia, growing in disturbed habitats and among grasses in full sunlight.



Authors: E. Hágsater, C. Rincón-Useche & O. Pérez Illustrators: C. Rincón-Useche & O. Pérez Photo: O. Pérez Editors: E. Hágsater & L. Sánchez S.

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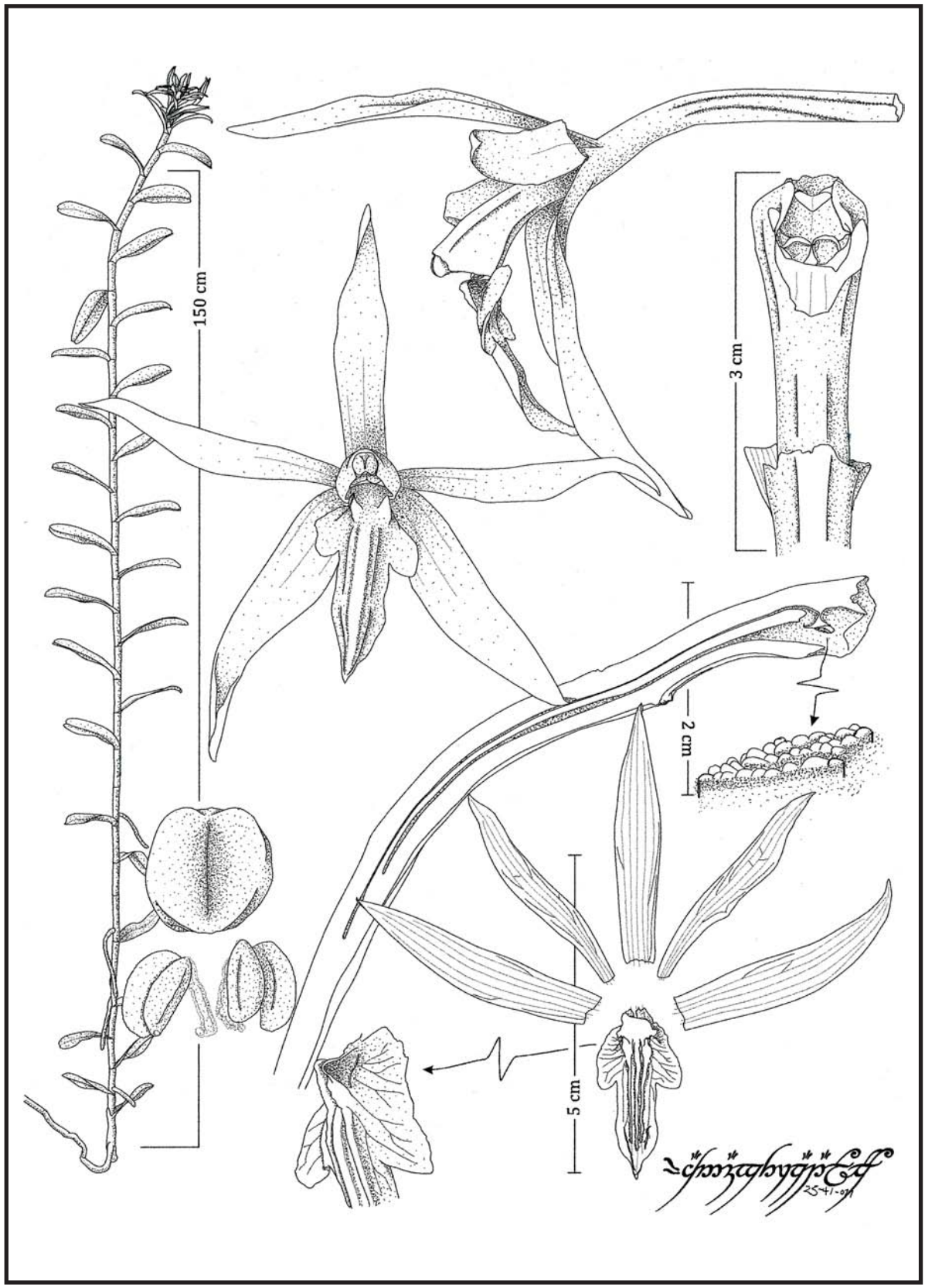


EPIDENDRUM GERLACHIANUM Hágsater, O.Pérez & E.Santiago **Plate 1431**

EPIDENDRUM GERLACHIANUM Hágsater, O.Pérez *et* E.Santiago, *sp. nov.*

Type: COLOMBIA: Cundinamarca: Municipio de Guasca; Páramo de Guasca, junto a la carretera que conduce a Ubalá, *ca.* 3100 m, *ca.* 7° C, 22 July 2011, ***Oscar Alejandro Pérez Escobar & Gustavo Morales 1104.*** Holotype: CUVC! (Illustration and photo voucher). Digital images of pretype, AMO!

Epidendro steyermarki A.D.Hawkes simile, sed inflorescentia corymbosa subsubessile, floribus majoribus, sepalis lanceolatis oblongis acuminatis marginibus revolutis, petalis linearis oblongis, callis prominentibus triangulis recedit.

Terrestrial, monopodial, erect **herb,** to 170 cm tall. **Roots** 2.7-7.5 mm in diameter, produced along the basal 1/3 of the stem, fleshy, thick, scarce. **Stems** cane-like, 163 x 0.56-1.2 cm, simple when young, branching near the apex with time, terete, erect, straight. **Leaves** numerous, 29 in the type, alternate, articulate, coriaceous, deciduous, similar in size and shape; sheaths tubular, 28-58 x 10.9-14.5 mm, striated and rugose, ochre-colored, somewhat tinged reddish; blade elliptic to lanceolate, 3.1-12.1 x 1.8-3 cm, apex rounded, short bilobed, margin entire, green with the margin tinged reddish, somewhat lustrous. **Spathaceous bract** lacking. **Inflorescence** (5 cm long including the flowers), apical, corymbose, flowering only once, erect, compact, few-flowered; peduncle 2 mm long, very short, obsolete, terete, thick, bare; rachis very short, 17 mm long,. **Floral bracts** 9.3 mm long, much shorter than the ovary, narrowly lanceolate, acute, amplexicaul. **Flowers** 6, successive, but eventually all open at one time, resupinate, yellowish green; the column and lip white including the calli; fragrance not registered. **Ovary** 39-40 mm long, teretes to slightly flattened ventrally, not inflated, unornamented. **Sepals** 41-44 x 9 mm, oblong-lanceolate, acuminate, aristate, free, spreading, fleshy, 5-veined, margin entire, somewhat revolute; lateral sepals obliquely fused to the basal part of the column, slightly oblique. **Petals** linear-oblong, 40-41 x 6-7 mm, free, spreading, slightly convex, acuminate, 3-veined, branching somewhat below the middle, margin entire, somewhat revolute. **Lip** united to the column, 3-lobed, 27 x 14 mm, base truncate, fleshy; bicallose, the calli prominent, triangular, laminar; provided with 3 smooth, elongate ribs which disappear before reaching the apex of the mid-lobe; lateral lobes widely reniform-truncate, 5 x 10 mm, spreading, short, the corners narrowly rounded, margin slightly erose; mid-lobe oblong, 15 x 7 mm, apex obtuse, the apical 1/3 slightly bent downwards towards the adaxial surface of the lip, apiculate, margin entire. **Column** 23 mm long, slightly arched, robust, widened towards the apex. **Clinandrium-hood** short, margin entire. **Anther** sub-spherical, the apical half exposed and surpassing the apex of the column, 4-celled. **Pollinia** 4, obovoid, laterally compressed, unequal, the inner pair slightly smaller; caudicles soft and granulose, as long as the pollinia. **Rostellum** apical, slit. **Lateral lobes of the stigma** prominent, occupying 1/3 of the stigmatic cavity, papillose. **Nectary** not inflated, minutely papillose, penetrating 2/3 of the ovary. **Capsule** not seen.

OTHER SPECIMENS: COLOMBIA: Cundinamarca: Nemocón, 2750 m, 21 IV 1968, *García Barriga 19379,* COL!

OTHER RECORDS: VENEZUELA: Táchira: Párarmo La Negra, photo published as *Epidendrum steyermarkii* (Morillo, 2011). *Morillo s.n.*, digital image, AMO!

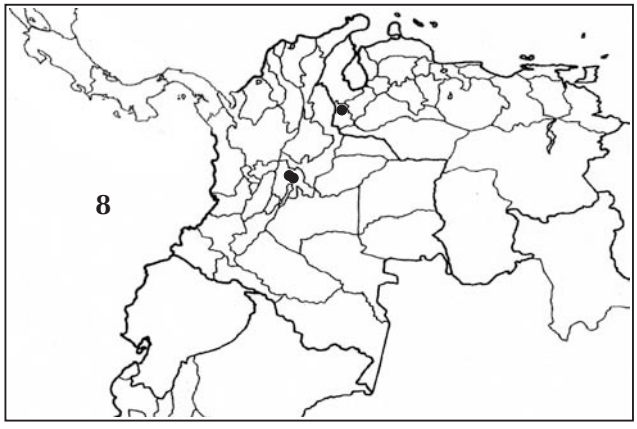
DISTRIBUTION AND ECOLOGY: presently known from the eastern Cordillera of the Andes, in Cundinamarca, Central Colombia, in the Páramo de Guasca, at 3100 m altitude and neighboring Venezuela; growing as a terrestrial along the road-side, in bush vegetation dominated mainly by *Befaria, Gaultheria* and *Chusquea* species.

RECOGNITION: *Epidendrum gerlachianum* belongs to the Andean Group and Cernuum subgroup, which is characterized by the monopodial, branching habit, the erect cane-like stems with a sub-apical branching, racemose, nutant inflorescence, compact, fleshy flowers, with the lip three-lobed. The species is recognized by the tall, erect, stem, erect, compact inflorescence, the large flowers with oblong-lanceolate sepals 41-44 mm long, linear-oblong petals 40-41 mm long, and the mid-lobe of the lip oblong, obtuse and apiculate, the apical 1/3 somewhat bent downwards towards the ventral surface of the lip, 15 mm long. It is similar to *Epidendrum steyermarkii* A.D.Hawkes, which has lax-flowered inflorescence, 5-7 cm long peduncle and rachis; large flowers with oblanceolate to oblong-elliptic sepals 26-35 mm long, oblanceolate to sub-spatulate petals 26-28 mm long, mid-lobe of the lip oblong and apiculate, 11-14 mm long. *Epidendrum pichinchae* Schltr. has taller plants, 2 m high, branched above, smaller flowers, the floral segments long-acuminate, lateral sepals falcate, [15]18-21 mm long, the mid-lobe of the lip sub-rhombic towards the apical half, with a long, parallel-sided isthmus, and a prominent apiculus.

CONSERVATION STATUS: DD. Data deficient.

ETYMOLOGY: In honor of Günter Gerlach (Germany, 1953-), Scientific Director of the living collection at the Botanische Garten München, in recognition of his contribution to the taxonomy and phytochemistry of the Subtribe Stanhopeinae. He is an authority of the genus *Coryanthes* and is currently working on the systematics os several general in Catasetinae and Zygopetalinae.

REFERENCES: Morillo, Gilberto, 2011, Familia Orchidaceae in Morillo, G., B. Briceño & J F. Silva (eds.) **Botánica y Ecología de las Monocotiledónes de los Páramos en Venezuela**. 1: 344, photo 28. Santiago, E., & E. Hágsater, 2009, *Epidendrum steyermarkii*, in The Genus *Epidendrum*, Part 8, **Species New and Old in *Epidendrum***, E. Hágsater & L. Sánchez Saldaña (eds.), **Icon. Orchid.** 12: t. 1294.



Authors: E. Hágsater, O. Pérez & E. Santiago **Illustrator: O. Pérez** **Photo: O. Pérez** **Editors: E. Hágsater & L. Sánchez S.**

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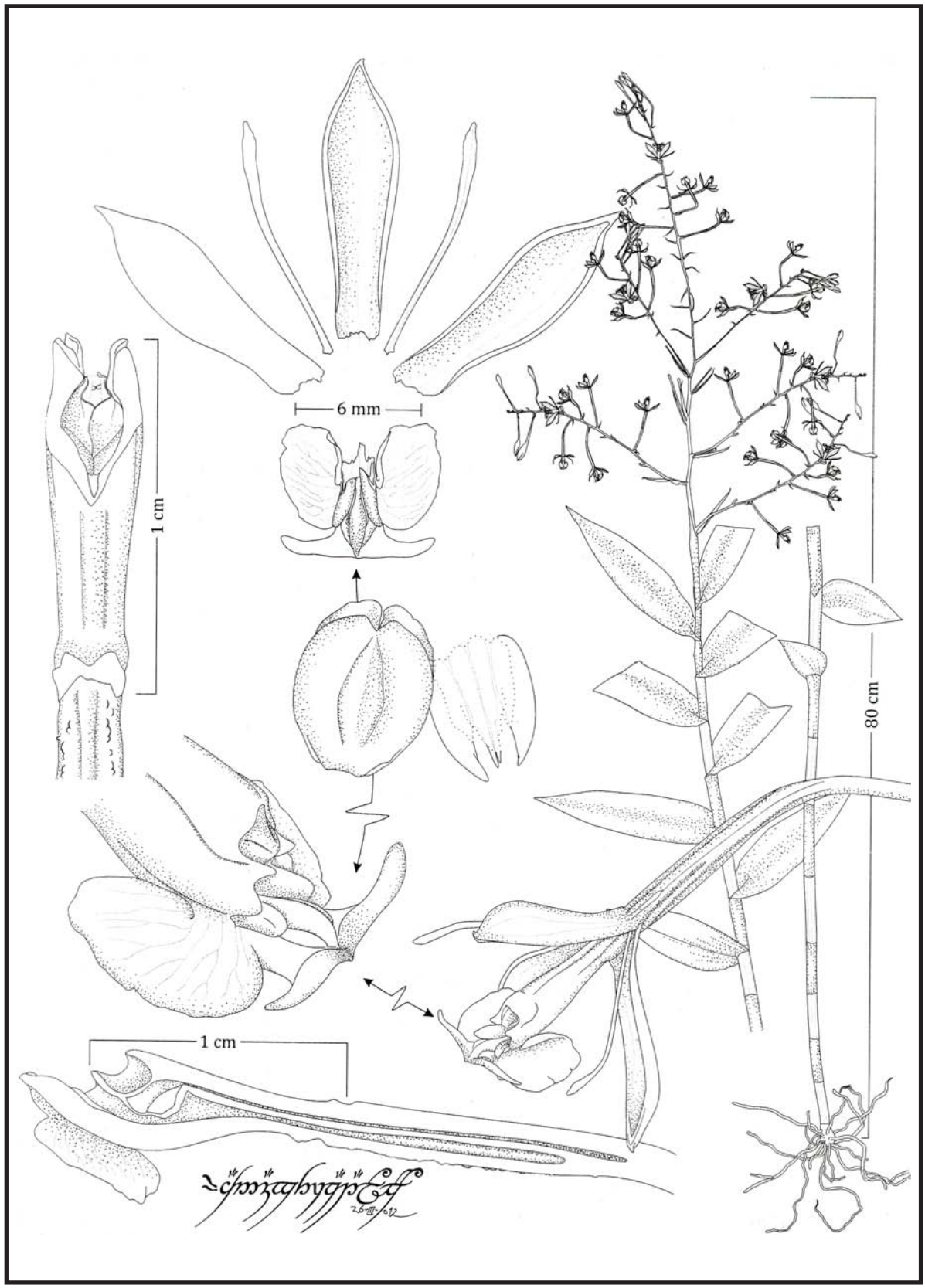


EPIDENDRUM GIRALDO-CANNASII Hágsater, O.Pérez & E.Santiago **Plate 1432**

EPIDENDRUM GIRALDO-CANNASII Hágsater, O.Pérez *et* E.Santiago, *sp. nov.*

Type: COLOMBIA: Valle del Cauca: Municipio de Yumbo; Dapa, ca. 1800 m, 15 Febrero 2011, ***Oscar Alejandro Pérez Escobar & Edicson Parra Sánchez 1103***. Holotype: CUVC! (Illustration voucher).

Similar to *Epidendrum peraltum* Schltr. but the leaves green, the underside purple, flowers delicately fragrant, sepals dorsally magenta, ventrally ochraceous, lip with the lateral lobes dolabriform, and the lobes of the mid-lobe straight, opposite.

Epiphytic, sympodial, caespitose **herb** up to 120 cm tall. **Roots** *ca*, 3 mm in diameter, basal, fleshy, white. **Stems** 77 x 1 cm, simple, cane-like, terete, straight, ascending; the basal 1/3 covered by non-foliar, tubular sheaths 3.1-5.2 cm long, minutely striated, scarious. **Leaves** 11, distributed along the apical 2/3 of the stem, alternate, articulate, spreading, unequal in size, the basal ones smaller; sheaths 24-70 x 3.5-10 mm, tubular, minutely striated, purple; blade 4.1-14.5 x 3-4 cm, elliptic, acuminate, coriaceous, the upper face green, the underside purple, margin entire, spreading. **Spathaceous bract** lacking. **Inflorescence** 40 cm long, apical, paniculate, flowering only once, laxly many-flowered, arched-nutant; peduncle 6 cm long, short, straight, thin, totally hidden by a single tubular bract 7.5 cm long, acuminate, amplexicaul; rachis 34 cm long, terete, gradually becoming thinner towards the apex, with some 8 few-flowers racemes 11-15 cm long, each subtended by a bract 2-4.5 cm long, linear-triangular, long-acuminate, amplexicaul. **Floral bracts** 3-17 mm long, small, much shorter than the ovary, narrowly triangular, acuminate, amplexicaul. **Ovary** 31 mm long, terete, striated, papillose, magenta colored, slightly inflated along the apical half, and bent at the middle into an angle of 135°. **Flowers** *ca*. 100, resupinate, most of them simultaneously at anthesis (only a few apical flowers in bud), sepals dorsally magenta, internally ochraceus, petals and lip light pink, column magenta, somewhat yellowish at base; fragrance diurnal, delicate, agreeable. **Sepals** 13.5 x 3.6 mm, partly spreading, free, oblanceolate, acute, apiculate, fleshy, slightly concave near the apex, 3-veined, margin entire, spreading; the lateral sepals slightly oblique. **Petals** 11 x 0.6 mm, partly spreading, free, filiform, slightly oblique, apex sub-acute, 1-veined, margin entire, spreading. **Lip** 6.4 x 7.5 mm, united to the column, 3-lobed, base cordate; bicallose, the calli thin, prominent, elongate to the base of the mid-lobe; disc provided with 3 fleshy, parallel ribs, the mid-rib reaching the apical sinus, the lateral pair shorter and lower; lateral lobes 3 x 5 mm, dolabriform, margin somewhat erose; mid-lobe 1.5 x 7 mm, forming a pair opposite lobes 3.5 x 0.6 mm, horn-like, falcate, apex narrowly rounded. **Column** 11 mm long, straight, thin at the base, gradually dilated towards the apical half, apex bidentate. **Clinandrium-hood** reduced, margin entire. **Anther** ovoid, 4-celled. **Pollinia** 4, bird-wing type; caudicles laminar, shorter than the pollinia. **Rostellum** apical, slit. **Lateral lobes of the stigma** not seen. **Nectary** penetrating nearly half of the ovary, not inflated, unornamented. **Capsule** not seen.

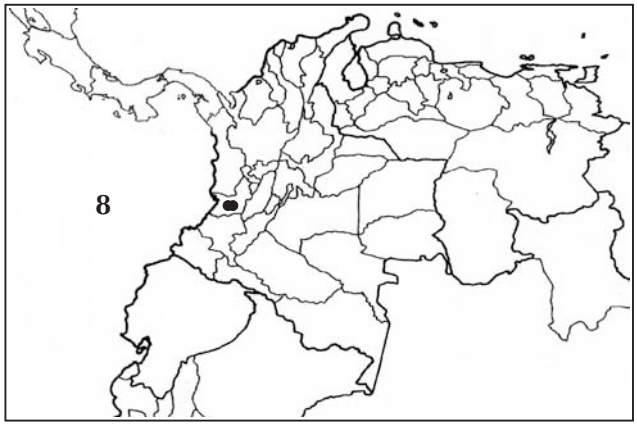
OTHER RECORDS: COLOMBIA: Valle del Cauca: Cali-Buenaventura, 2000-2050 m, col. 24 IV 1983, pressed cult. 28 VI 1992, *Hágsater 7306*, AMO! (Illustration, AMO!)

DISTRIBUTION AND ECOLOGY: Endemic to southern Colombia. Known from the eastern slope of the Cordillera Occidental of the Andes. Grows as epiphyte in cloud forests, at ca. 2000 m. Flowering in February to April.

RECOGNITION: *Epidendrum giraldo-canasii* belongs to the Pseudepidendrum Group, which is characterized by caespitose plants, cane-like stems, acute to acuminate leaves, apical inflorescence without spathaceous bract, the petals filiform and the lip usually 3-lobed, with 3 parallel fleshy ribs, the apical lobe often bifurcate, and the pollinia "bird-wing type" and Porphyreum Subgroup which has flowers colored reddish orange, deep purple or lilac-pink, the calli generally prominent, sometimes horn-like. The species is recognized by the intense purple color of the lower surface of the leaves, stems stained with purple, the prominent calli of the lip elongated until the base of the mid-lobe, lateral lobes of the lip dolabriform, 3 x 5 mm, and by the hastate, linear lobes of the mid-lobe, strongly divaricated, as long as the lateral lobes. It is similar to *Epidendrum peraltum*, which has larger plants, green, concolor leaves, and somewhat larger yellowish-green, un-fragrant flowers tinged with pink or totally dirty pink, lateral lobes of the lip falcate-oblong, strongly retrorse, and the mid-lobe with a short isthmus, ended in a pair of lobes strongly divaricated shorter than the lateral lobes. *Epidendrum capricornu* Kraenzl. endemic to Peru and Ecuador, has shorter plants, sepals with the outer surface purple brown, the inner surface yellow, slightly wider between the lateral lobes of the lip than between the apical lobes, and the mid-lobe strongly emarginate, forming a pair of linear-horn-like lobes, slightly divaricate.

CONSERVATION STATUS: DD. Data deficient.

ETYMOLOGY: Named after Dr. Diego Giraldo Cañas, professor and researcher of the Universidad Nacional de Colombia (Bogotá) at the Instituto de Ciencias Naturales, (ICN), who has greatly contributed to the knowledge of the systematic of the families Marcgraviaceae and Poaceae, through the publication of several scientific papers and books. He was an advisor of O.Pérez during his undergraduate studies.



Authors: E. Hágsater, O. Pérez & E. Santiago **Illustrator: O. Pérez** **Photos: O. Pérez** **Editors: E. Hágsater & L. Sánchez S.**

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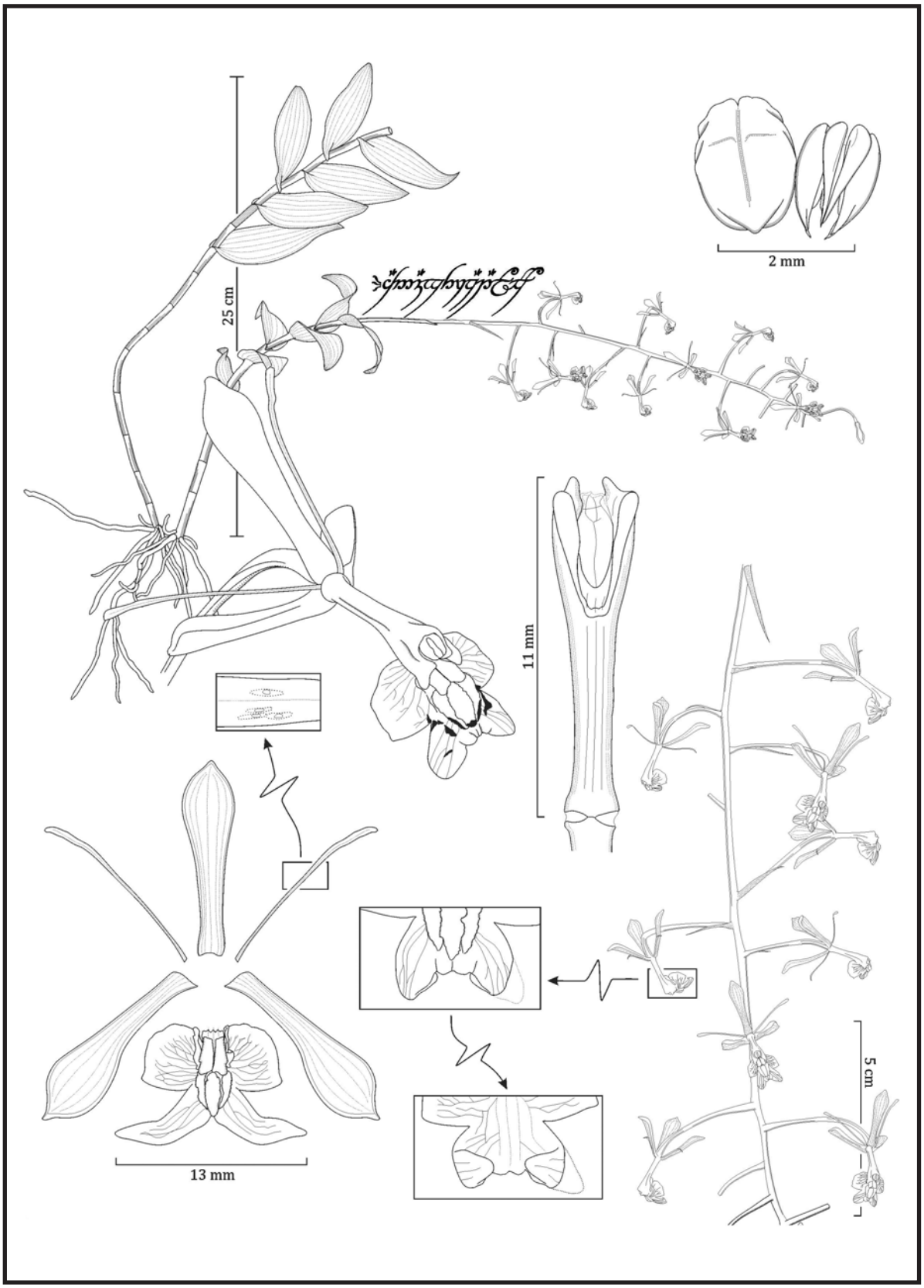


EPIDENDRUM KOLANOWSKAE Hágsater, O.Pérez & E.Santiago **Plate 1447**

EPIDENDRUM KOLANOWSKAE Hágsater, O.Pérez *et* E.Santiago, *sp. nov.*

Type: COLOMBIA: Valle del Cauca: Municipio de Yumbo; Dapa, en frente de la Finca "Cielo Azul", 1900 m, ca. 20° C, 1 August 2010, ***Oscar Alejandro Pérez Escobar 821***. Holotype: CUVC! (Illustration voucher).

Similar to *Epidendrum paniculorotundifolium* Hágsater, M.Kolanowska & E.Santiago, but the leaves variable in shape from orbicular to elliptic (even on the same stem), and the disc of the lip with purple markings at the base of the lobes, surrounding the ribs.

Epiphytic or lithophytic, sympodial, caespitose, decumbent **herb**, ca. 42 cm tall**. Roots** 2-3 mm in diameter, basal, fleshy, thin. **Stems** simple, cane-like, 16-30 x 0.3-0.6 cm, terete, straight; the basal half covered by non-foliar, tubular sheaths 1.8-2.5 cm long **Leaves** 7-13, distributed throughout the apical half of the stem, alternate, articulate, erect-spreading, amplexicaul, some unequal in size, green and concolor when young, the underside turning purple when mature; sheath tubular, 1.8-2.0 x 0.3-0.6 cm, minutely striated, green; blade orbicular to elliptic, 2.6-7.5 x 1.8-2 cm, acute, sub-coriaceous, margin entire, spreading. **Spathaceous bracts** lacking. **Inflorescence** apical, 26 cm long, racemose or paniculate, flowering only once, lax, few-flowered; peduncle 7 cm long, elongate, straight, thin, provided with 2 lanceolate, acuminate, amplexicaul bracts, 8 mm long; rachis 19 cm long, when paniculate, with a spreading basal, short, few-flowered branch subtended by a basal narrowly triangular, acuminate, amplexicaul bract. **Floral bracts** 12-20 mm long, prominent, unequal in size, the basal ones about half as long as the ovary, the apical ones 1/4 the length of the ovary, linear-triangular, acuminate, amplexicaul. **Ovary** 25-28 mm long, terete, thin, not inflated. **Flowers** 8-17, simultaneous, resupinate, sepals, petals and basal half of the column green, lip and apical half of the column with the disc surrounded by pale purple spots spilling onto the lobes of the lip. **Sepals** 13 x 3.5 mm, reflexed, free, oblanceolate-spatulate, obtuse, fleshy, slightly concave towards the apex, 4-5-veined, margin entire, spreading. **Petals** 13 x 0.3 mm, reflexed, free, filiform, slightly falcate, 1-veined, apex rounded, margin entire, spreading (illustration insert shows large size of cells). **Lip** united to the column, 8.3 x 13 mm, slightly convex, fleshy, 3-lobed, base cordate, margin entire; bicallose, the calli prominent, rectangular-cubical, disc provided with 3 parallel ribs which extend to apical sinus, fleshy; lateral lobes 3.6 x 4.6 mm, dolabriform; mid-lobe bilobed, divaricate, lobes linear-oblong, oblique, apex acute, revolute, each lobe 6.6 x 2.3 mm. **Column** 11 mm long, straight, thin at the base, dilated towards the apical half. **Clinandrium-hood** reduced, margin entire. **Anther** ovoid, 4-celled. **Pollinia** 4, bird-wing type, the inner pair somewhat smaller, caudicles laminar, shorter than the pollinia. **Rostellum** apical, slit. **Lateral lobes of the stigma, nectary** and **capsule** not seen.

OTHER SPECIMENS: COLOMBIA: Without locality: Cult. Colomborquídeas, col. 1 VII 1992, press. 22 VII 1997, *Hágsater 11668*, AMO! (spirit & slide). **Antioquia:** Fredonia: Cerro Bravo, 1770-2050 m, 6 VI 1992, *Fonnegra 4384*, COL! HUA! NY! **Valle del Cauca:** Municipio de Argelia, vereda "Las Brisas", 1950 m, 22 I 1983, *Franco 1746*, COL! Versalles, 2 VII 2012, *Rincón-Useche & Ríos 43*, COL!

OTHER RECORDS: COLOMBIA: Valle del Cauca: Municipio de Yumbo; Dapa, 21 XII 2010, *Pérez 1023*, digital image, VALLE! AMO! Versalles, 2 VII 2012, *Rincón-Useche & Ríos 43*, digital image, AMO!

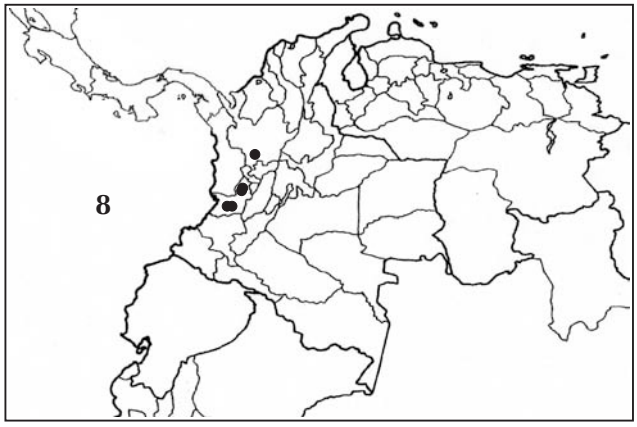
DISTRIBUTION AND ECOLOGY: Known from the forested summit of the Cordillera Occidental in Colombia, in the municipality of Yumbo, Valle del Cauca, and the Cordillera Central near Medellín, Antioquia. Epiphytic or lithophytic in conserved cloud forest at ca. 1770-2050 m, altitude. Flowering from June to December.

RECOGNITION: *Epidendrum kolanowskae* belongs to the Pseudepidendrum Group, which is characterized by caespitose plants, cane-like stems, acute to acuminate leaves, apical inflorescence without spathaceous bract, the petals filiform and the lip usually 3-lobed, with 3 parallel fleshy ribs, the apical lobe often bifurcate, and the pollinia "bird-wing-type", and Paniculatum Subgroup, which has bicolor flowers (generally green with the lip and apex of the column white), the disc sometimes marked with purple to red. The species is recognized by the intermediate sized plants, the racemose or paniculate inflorescence, leaves orbicular to elliptic, disc of the lip with 3 ribs, surrounded by pale purple spots spilling onto the lobes. *Epidendrum weerakitianum* Hágsater, O.Pérez & E.Santiago always has elliptic leaves; sepals 3-veined, the lip with 5 ribs on the disc which is clearly marked with purple turning reddish-purple with age. *Epidendrum paniculorotundifolium* has orbicular-elliptic leaves, sepals 5-veined, and the disc of the lip also has 3 ribs, but the lip is immaculate. It is similar to *Epidendrum paniculatum* Ruiz & Pav. which has elliptic leaves, the lip marked with reddish-purple, the lateral lobes of the lip sub-orbicular and the mid-lobe bilobed, formed by a pair of strongly divaricate, falcate lobes; it is endemic to NW Peru.

CONSERVATION STATUS: DD. Data deficient.

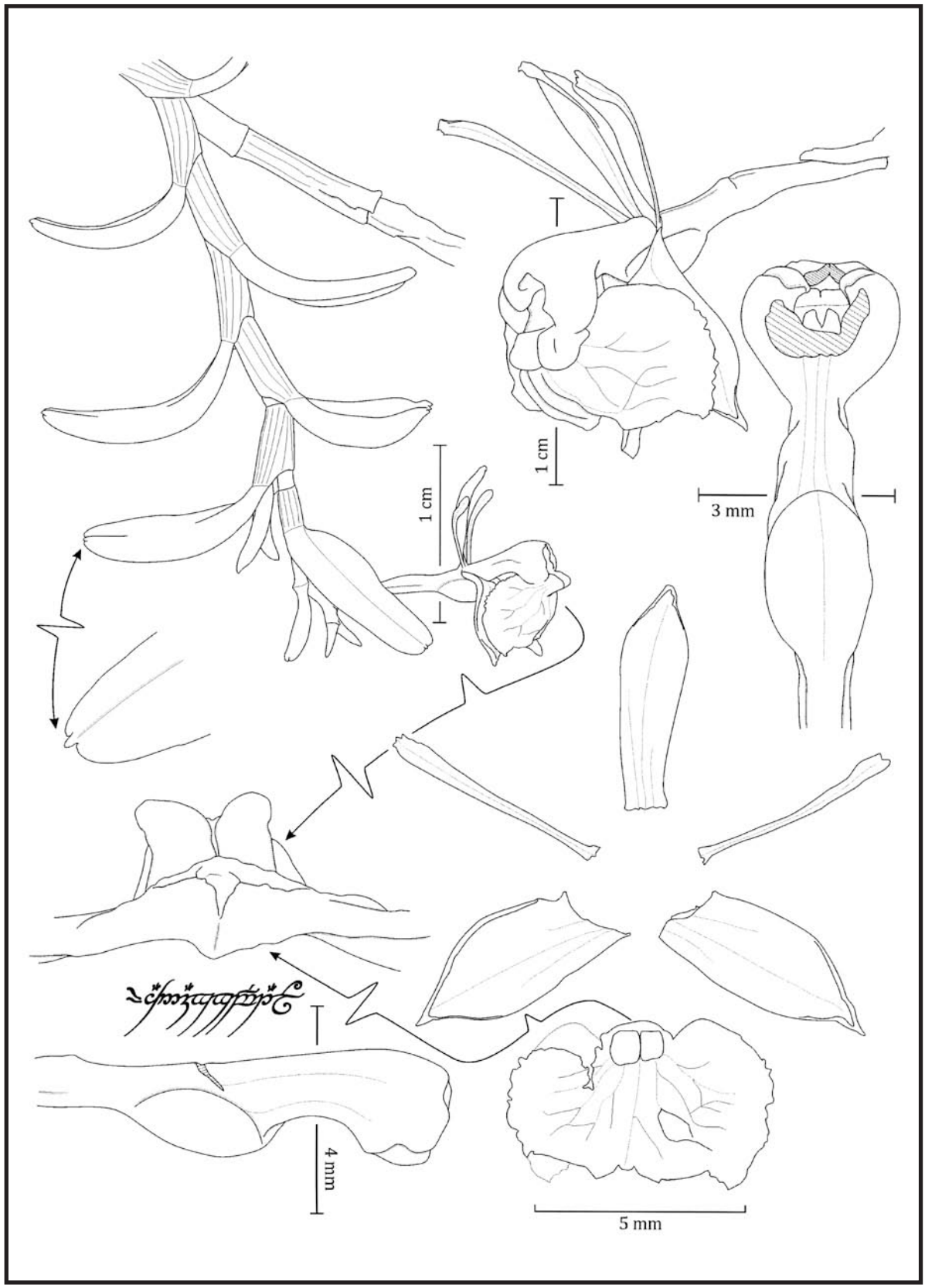
ETYMOLOGY: In honor of Marta Kolanowska, a Ph.D. student of the University of Gdansk (Poland) who has worked for the past 3 years in the study of the orchid flora of Valle del Cauca (Colombia), and recently authored (Kolanowska et al., 2011) An Illustrated field guide to the orchids of the Yotoco Forest Reserve (Colombia).

REFERENCE: Kolanowska, Marta, Oscar Alejandro Pérez Escobar, Edicson Parra Sánchez & Dariusz L. Szlachetko, 2011, **An Illustrated field guide to the orchids of the Yotoco Forest Reserve (Colombia)**, Gdansk, Poland.



Authors: E. Hágsater, O. Pérez & E. Santiago **Illustrator: O. Pérez** **Photos: O. Pérez** **Editors: E. Hágsater & L. Sánchez S.**

Herbario AMO **México, D.F. MÉXICO** **ICONES ORCHIDACEARUM 14. 2013.** **Plate 1447**



EPIDENDRUM LEONORAE Hágsater, O.Pérez & E.Santiago **Plate 1448**

EPIDENDRUM LEONORAE Hágsater, O.Pérez *et* E.Santiago *sp. nov.*

Type: COLOMBIA: Valle del Cauca: Municipio de Yumbo; Dapa, growing on a dead branch of a Melastomataceae tree, 2000 m, 18°C, 2 December 2010, ***Oscar A. Pérez E. & Julián González 1000.*** Holotype: VALLE! (Illustration and photo voucher).

Simile *Epidendri moscozoi* Hágsater & E.Santiago sed floribus albo-viridaceis roseo suffusis, ovario inflato a tergo perianthi, sepalis 3-nervatis, sepalo dorsali extenso, apice labelli emarginato et minute apiculato geminis lobulis semicircularibus externe dentatis.

Epiphytic, monopodial, reclined, branching **herb**, the main stem 9.1-15 cm tall. **Roots** 1.4 mm in diameter, basal, from the main stem, fleshy, thin. **Stems** branching; the main stem 8.3-14 x 0.14 cm; the branches 2.5-3.5 x 0.18 mm; cane-like, terete at the base, slightly laterally compressed towards the apex, very thin. **Leaves** 6 on the main stem, 4-6 on the branches, distributed throughout the stems, alternate, articulate; sheath 1-7.5 x 1.4-1.8 mm, somewhat infundibuliform, striated; blade 5-17 x 1.5-3.5 mm, linear-lanceolate, obtuse, minutely apiculate, margin entire, sub-coriaceous. **Spathaceous bract** lacking. **Inflorescence** 4-9.5 mm long, apical, racemose, short, 2-flowered. **Floral bract** 1.7-2 mm long, shorter than the ovary, triangular, acuminate, amplexicaul. **Flowers** 2, resupinate, greenish-white, with tinged pink to purple at the base of the petals and mid-part of the column and anther; without fragrance. **Ovary** 8 mm long, terete, thin, smooth, ventrally inflated behind the perianth to form a vesicle. **Sepals** 3-veined, margin entire, spreading, free; the **dorsal** sepal 5.5 x 2 mm, sub-spatulate, obtuse; the **lateral** sepals 5.5 x 2.5 mm, obliquely elliptic, acute, with a prominent low dorsal keel. **Petals** 5.5 x 0.5-0.7 mm, spreading, free, linear-filiform, slightly expanded towards the apex, 1-veined, margin slightly erose at the apex, spreading. **Lip** 4 x 6.5 mm, united to the column, markedly convex, transversely elliptic, base slightly cordate, apex emarginate, apiculate, flanked by a pair of semi-circular small lobes terminated in a pair of prominent teeth on the outer margin, margin erose-crenate; bicallose, the calli, globose, large; disc fleshy. **Column** 5 mm long, straight, narrowed in the middle. **Clinandrium-hood** short, margin entire. **Anther** and **pollinia** not seen. **Rostellum** apical, slit. **Lateral lobes of the stigma** not seen. **Nectary** not seen. **Capsule** 22-24 x 8-9 mm, globose; pedicel 3-4 mm long, terete; short, thin; body 12-14 x 8.5-9 mm; apical neck 6-7 mm long.

OTHER SPECIMENS: PERU: Huánuco: San Pedro Carpish, 2755 m, 18 II 2007, Trujillo 354, HURP! (Flowers in spirit: MOL; Illustration, Photos AMO!)

OTHER RECORDS: COLOMBIA: Antioquia: Serranía de Las Baldías, Corregimiento de San Félix, Municipio de Bello, 2900 m, 27 I 2012, *Calderón-Franco & Zuleta s.n.* Digital series, AMO!

DISTRIBUTION AND ECOLOGY: Known from the forested summit of the Cordillera Occidental in the municipality of Yumbo, Valle del Cauca, Colombia, and the Cordillera Central north of Medellín, Antioquia, as well as Carpish, Huánuco, Perú. Epiphytic in disturbed cloud forest in regeneration process, growing on *Tibouchina lepidota* (Bonpl.) Baill., at 2000-2900 m altitude. Flowering in December and February.

RECOGNITION: *Epidendrum leonorae* belongs to the Soratae Subgroup of the Scabrum Group which is characterized by the branching habit starting on a monopodial, primary stem, infundibuliform, rugose leaf-sheaths, lanceolate, aristate, acute leaves, racemose sub-capitate inflorescence on a short, thin peduncle, and the bicallose lip. The species is recognized by the small plants with thin stem and very short branches, linear-lanceolate, short leaves, small greenish-white flowers, lateral sepals obliquely elliptic, 5.5 mm long, and a transversely elliptic lip, base slightly cordate, apex emarginate, apiculate, the apiculus flanked by a pair of semi-circular lobes terminating in a pair of prominent teeth at the outer margin, the margin crenate. It is similar to *Epidendrum moscozoi*, which has white flowers with 1-veined, ovate lateral sepals (the dorsal one reflexed) 3.7-4 mm long, and a reniform lip with a unicarinate disc, the ovary is 10-14 mm long. *Epidendrum obliquum* Schltr. has plants to 57 cm tall and a reniform, emarginate lip with entire margin, the ovary is not inflated. *Epidendrum soratae* Rchb.f. has taller plants (to 27 cm), yellowish-green flowers with sepals 6.8-7 mm long, and a reniform, emarginate, somewhat 3-lobed lip with the base deeply cordate, and margin entire.

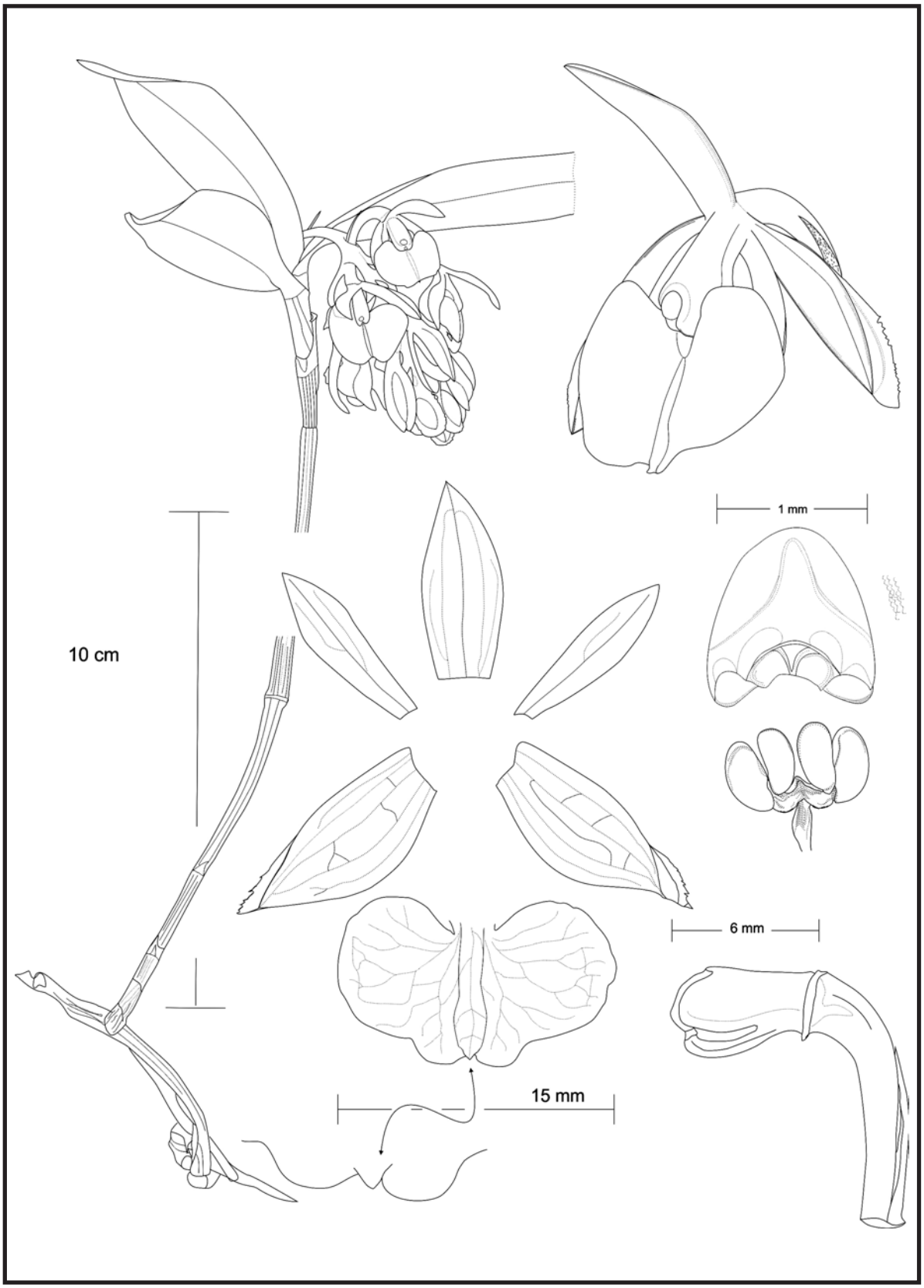
CONSERVATION STATUS: DD. Data deficient.

ETYMOLOGY: Named after Leonor Escobar Sora, mother of the second author, in recognition to her unconditional support given for his academic formation.

ACKNOWLEDGEMENT: We wish to thank Diego Calderón-Franco and Julián Zuleta, ornithologists from Medellín, for sharing their information, images, and the sighting of this new species near Medellín.

Authors: E. Hágsater, O. Pérez & E. Santiago Illustrator: O. Pérez Photo: O. Pérez Editors: E. Hágsater & L. Sánchez S.

Herbario AMO México, D.F. MÉXICO ICONES ORCHIDACEARUM 14. 2013. Plate 1448



EPIDENDRUM LUIS-SANCHEZII Hágsater, E.Parra & O.Pérez **Plate 1452**

EPIDENDRUM LUIS-SANCHEZII Hágsater, E.Parra *et* O.Pérez, *sp. nov.*

Type: COLOMBIA: Valle del Cauca: Cerrito, Tenerife, 3500 m, 6 de Febrero de 2011, ***Edicson Parra Sánchez 381.***
Holotype: VALLE! (Illustration voucher).

Simile *Epidendri aylacotoglossi* Hágsater sed foliis ovato-ellipticis, labello obscure trilobato, ecalloso, disco praedito carina lata et humili attingente apicem laminae; petalorum apices acuti et columna recta.

Epiphytic, sympodial, erect **herb**, 20 cm tall or more, where the new stem originates from a sub-apical internode of the previous stem. **Roots** produced from the base of the primordial stem, thick. **Stems** 16.5 x 0.4 cm, terete, the new stem produced from a sub-apical internode of the previous stem, below the leaves; the basal ¾ covered by tubular, non-foliar, striated, scariose sheaths, 1.3-3.8 cm long. **Leaves** 3, aggregate towards the apex of the stem, unequal in size, subcoriaceous, alternate, articulate; sheaths 1.4 x 0.5 cm, tubular, striated; blade 6-9 x 2.5-3 cm, obovate-elliptic, obtuse, margin entire. **Spathaceous bract** lacking. **Inflorescence** 5.4 cm long, apical, flowering only once, densely few-flowered; peduncle 1.6 cm long, terete, thin, straight, short. **Floral bracts** 7.5 mm long, half as long as the ovary, triangular, acuminate, amplexicaul. **Flowers** 10-15, fleshy, successive, though several are open at one time, yellow, the ovary greenish yellow, darker towards the base; without fragrance. **Ovary** 12 mm long, terete, arched near the apex, not inflated, somewhat grooved. **Sepals** partly spreading to spreading, free, obovate, acute, 3-5-veined, margin entire, spreading; the **dorsal** sepal 11 x 4.5 mm; the **lateral** sepals 13 x 5.5 mm, oblique with a prominent, serrulate, awned dorsal keel. **Petals** 10.5 x 2.8 mm, partly spreading, the apical 2/3 hidden beneath the lip in natural position, oblanceolate, acute, 1-veined, the vein branched around its mid-point, margin entire, spreading. **Lip** 9 x 15 mm, united to the column, obscurely 3-lobed, widely reniform, base cordate, concave in front of the column, the rest of the lip convex, margin entire; ecallose, the disc with a wide, low rib spreading from the base to the apex of the lip; lateral lobes 7.0 x 7.5 mm, semi-orbicular; mid-lobe 1.4 x 6 mm, short, transversely rectangular, the apex emarginate, minutely apiculate, forming a pair of small, rounded lobes. **Column** 6 mm long, straight, short, thick; the apex with a pair of prominent rounded wings. **Rostellum** apical, slit. **Lateral lobes of the** stigma not seen. **Clinandrium-hood** reduced, margin entire. **Anther** 1 mm wide, obovoid, ornamented, papillose, 4-celled. **Pollinia** 4, obovoid; caudicles as long as the pollinia. **Rostellum** sub-apical, slit. **Nectary** and **capsule** not seen.

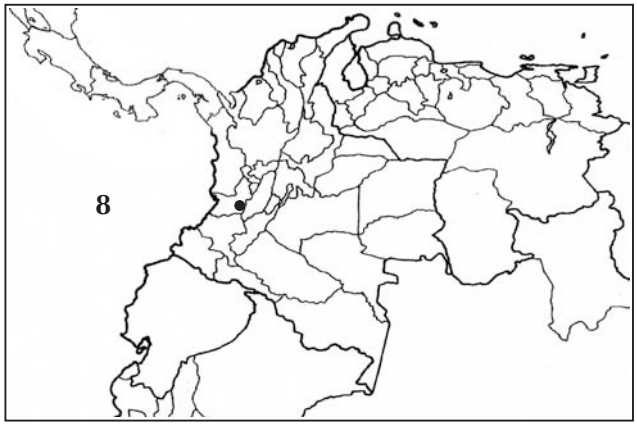
OTHER SPECIMENS: None seen.

DISTRIBUTION AND ECOLOGY: Endemic to southern Colombia, on the western slope of the Central Cordillera of the Andes. So far known only from the remnant paramune vegetation of the Cerrito Municipality, Tenerife village, Valle del Cauca. Epiphytic in secondary humid cloud forest, at 3500 m altitude. Flowering in March.

RECOGNITION: *Epidendrum luis-sanchezii* belongs to the Arbuscula Group which is characterized by the erect habit with successive lateral growths produced from the middle of the previous growth, few leaves aggregate towards the apex of the stems, roots generally only from the base of the primordial stem, and the Incomptum Subgroup which has a short apical inflorescence with fleshy yellowish to green to violet-green to black flowers with short ovaries, the lip entire to 3-lobed. The species is recognized by obovate-elliptic leaves, the yellow flowers, sepals with a prominent, acute and serrulate keel on the ventral side, the apical ¾ of the petals hidden beneath the ecallose lip which is obscurely 3-lobed, the mid-lobe emarginate, apiculate, forming a pair of rounded lobes, with a low thick rib running the length of the lip. *Epidendrum aylacotoglossum* Hágsater has elliptic leaves, the clearly 3-lobed lip with a pair of low calli and a short canal in the middle, and the column sigmoid. *Epidendrum envigadoënse* Hágsater has narrow, lanceolate leaves, a wider lip (17.5-19 mm) with a pair of small calli and the apex emarginate, not apiculate. *Epidendrum amayense* Hágsater has linear-lanceolate leaves (0.5-0.6 mm wide), somewhat smaller, green flowers, the sepals (9 mm long) with a low dorsal keel, and the lip without any thickened rib. *Epidendum oligophyllum* F.C. Lehm. & Kränzl. is vegetatively very similar, with oblong-elliptic to elliptic leaves, olive-brown flowers, the lip bicallose and the column 10 mm long. *Epidendrum morae* P.Ortiz, Hágsater & L.E.Álvarez has elliptic leaves, pale yellow flowers somewhat tinged pink-violaceous, the sepals have no dorsal keel, and the 3-lobed lip is bicallose with 3 low ribs on the disc.

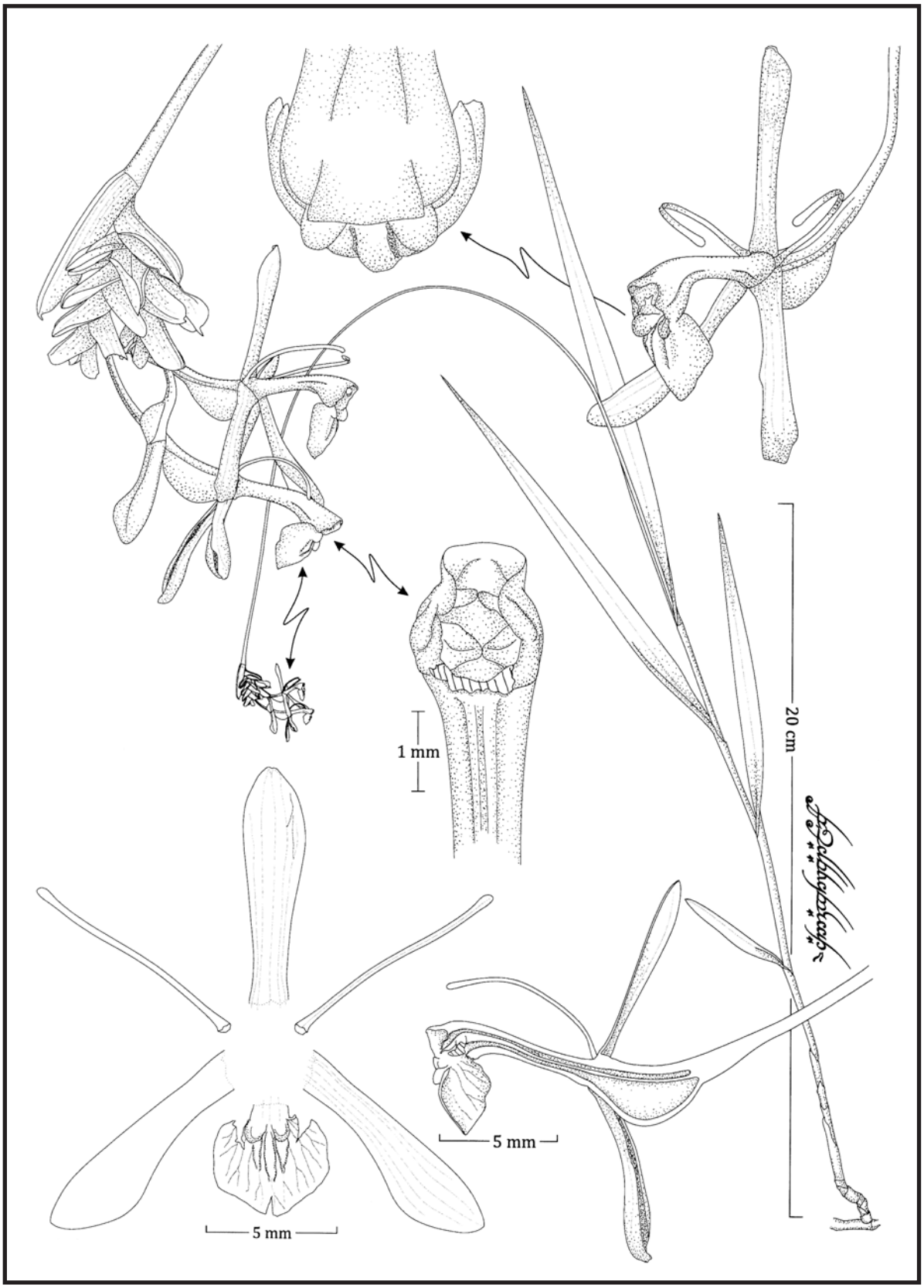
CONSERVATION STATUS: DD. Data deficient.

ETYMOLOGY: In honor of Luis M. Sánchez Saldaña, Mexican orchid researcher at the AMO Herbarium, who has contributed to the taxonomy of neotropical orchids, for his support in the research of native Colombian orchids.



Authors: E. Hágsater, E. Parra & O. Pérez **Illustrator: E. Parra** **Photo: E. Parra** **Editors: E. Hágsater & L. Sánchez S.**

Herbario AMO **México, D.F. MÉXICO** **ICONES ORCHIDACEARUM 14. 2013. Plate 1452**



EPIDENDRUM MACROPHYSUM Hágsater, O.Pérez & E.Santiago **Plate 1453**

EPIDENDRUM MACROPHYSUM Hágsater, O.Pérez *et* E.Santiago *sp. nov.*

Type: COLOMBIA: [Chocó]: Municipio de San José del Palmar, El Tabor, *ca.* 1400 m, 13 November 2011, ***Oscar Alejandro Pérez-Escobar & Duvan García-Ramírez 1400***. Holotype: CUVC! (Illustration voucher) Isotype: VALLE!

Similar to *Epidendrum jefeallenii* Hágsater & García-Cruz but the leaves narrower, the peduncle filiform, racemes of the inflorescence shorter, the margin of the sepals revolute and the ovary forming a very prominent vesicle, disc of the lip with 3 ribs.

Epiphytic, caespitose, sympodial **herb**, 40 cm tall. **Roots** basal fleshy. **Stems** 16.8-20 x 0.1-0.28 cm, cane-like, simple, straight, thin, basal half terete, ancipitose towards the apex. **Leaves** 4-5, distributed along the apical half of the stem, the basal one smaller, green, concolor; sheaths 2.9-4.45 x 0.12-0.28 cm, tubular, ancipitose, striated; blade 3-15.4 x 0.2-1 cm, linear-lanceolate, long-acuminate, with a central vein and a pair of evident secondary veins on the upper face, margin entire. **Spathaceous bract** 1.1 cm long, single, at the apex of the peduncle, narrowly elliptic, obtuse, conduplicate, ancipitose, similar to the floral bracts but larger. **Inflorescence** 20.5 cm long, apical, pluri-racemose, arching pendant, producing up to 4 racemes from within the apex of the peduncle; peduncle 19 cm long, filiform, ancipitose, two-winged, progressively narrower, the wings notorious at the base, thin; racemes ca. 1 cm long, short, densely few-flowered, rachis totally hidden by the floral bracts. **Floral bracts** 4-4.6 x 3.2-3.4 mm, shorter than the ovary, the basal ones sub-oblong, the apical ones elliptic, the apex rounded to minutely apiculate, conduplicate, dorsally carinate, distichous, imbricated at the base, persistent, pale green with small irregular, lilac spots. **Flowers** successive, 1 at a time per raceme, resupinate, pale green, the apex of the column greenish white at the height of the clinandrium-hood, callus and disc greenish white, the vesicle of the ovary and ovary with small, irregular lilac spots. **Ovary** 15.5 mm long, terete, thin, strongly inflated at the apex, forming a prominent ventricose vesicle. **Sepals** 9 x 2.4 mm, spreading, free, spatulate-lanceolate, sub-obtuse, 5-veined, margin entire, revolute; the lateral sepals oblique. **Petals** 9 x 0.4 mm, inflexed, free, linear, apex rounded, 1-veined, margin spreading, entire. **Lip** 3.9 x 4.5 mm, united to the column, entire, convex, sub-orbicular, cordiform when spread; bicallose, the calli prominent, fleshy, laterally compressed; disc with 3 fleshy, thin, parallel ribs, the surface rugose, the central rib slightly longer than the outer pair, though without reaching the apex of the lip. **Column** 7.2-7.6 mm long, thin along the basal 2/3, gradually widened towards the apex, slightly arched. **Clinandrium-hood** prominent (though without surpassing the body of the column, margin entire. **Rostellum** sub-apical, slit. **Lateral lobes of the stigma** prominent, covering half the stigmatic cavity. **Nectary** penetrating *ca.* 1/3 of the ovary, inflated, unornamented. **Anther** ca. 1 mm wide, 4-celled, transversely elliptic. **Pollinia** 4, slightly laterally compressed, sub-lenticular. **Capsule** 20 x 7.3 mm, ellipsoid, green with lilac spots on the pedicel and body, pedicel 6 mm long, body 11 x 7.3 mm, apical neck 3 mm long.

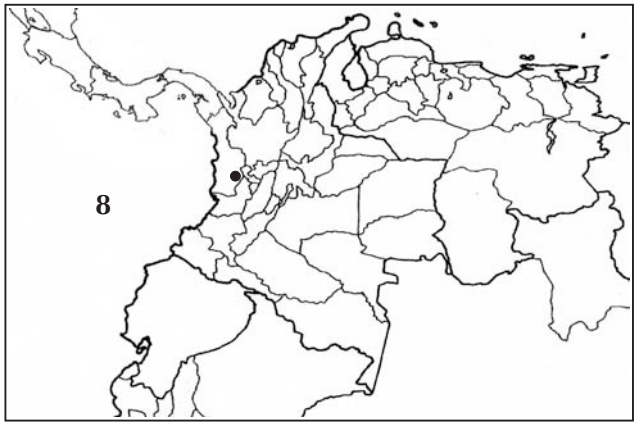
OTHER SPECIMENS: None seen.

DISTRIBUTION AND ECOLOGY: So far know from the western slope of the western range of the Los Andes, Department of Chocó, municipality of San José de El Palmar. Grows as an epiphyte in cloud forests at 1400-1500 m, where the populations are abundant.

RECOGNITION: *Epidendrum macrophysum* belongs to the Albertii Group which is characterized by the sympodial habit, laterally compressed to ancipitose or somewhat fusiform-thickened stems, the apical or apical and lateral racemose, distichous inflorescence more or less with imbricating bracts on the peduncle, producing one flower at a time, and the Allenii Subgroup which is characterized by the stems with numerous leaves, the inflorescence apical (rarely lateral), peduncle elongated, bare, non-bract bearing, two-winged, the rachis short, covered by rounded, usually imbricating bracts. The species is recognized by the almost filiform and long peduncle of the inflorescence, which is almost as long as the stem, the sub-oblong to elliptical, rounded floral bracts of 4-4.6 mm long, which somehow are a reminiscent of the glumes' flowers of some *Fimbristylis* species (Cyperaceae) and other sedges, the ventricose, prominent vesicle of the ovary and by the sub-orbicular to cordiform lip with a disc with three sub-equal ribs, 3.9 x 4.5 mm. It is similar to *E. jefeallenii* Hágsater & García-Cruz from Panama, which has more ancipitose peduncles, two-winged, the wings prominent; the inflorescence with larger racemes with 5-10 successive flowers; ovary slightly inflated in the apical third and a cordiform lip with emarginate apex, 4-5 x 5-6 mm, with a single rib spreading from the base to the half of the lip. It is also similar to *Epidendrum adnatum* Ames & C.Schweinf., from Costa Rica and Panama, which has shorter inflorescences with ancipitose peduncles, two-winged, the wings prominent towards the base; an ovary dilated just behind the perianth; oblanceloate, sub-acute, mucronate, sepals; and a ovate, sub-acute, apiculate lip with the margin slightly erose.

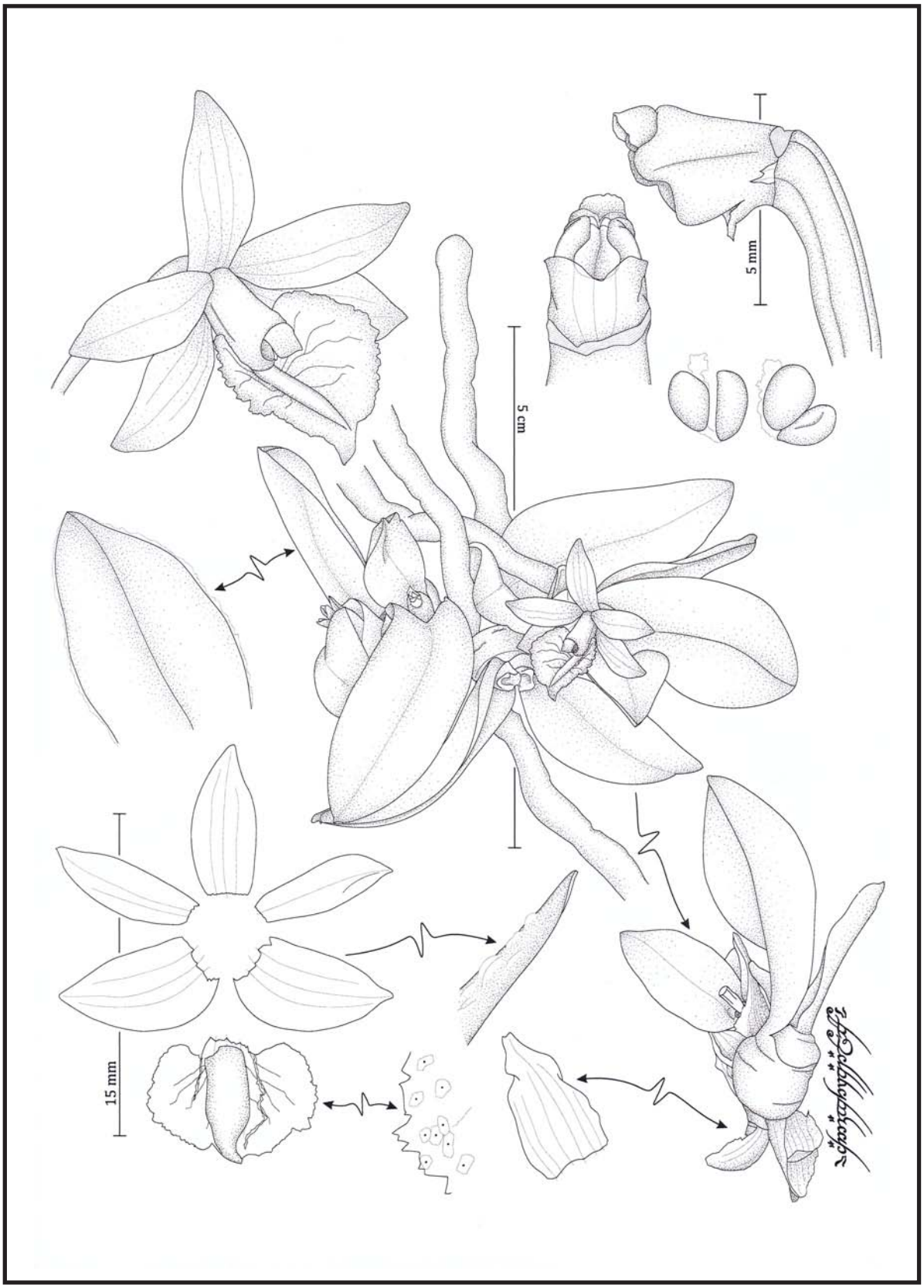
CONSERVATION STATUS: DD. Data deficient.

ETYMOLOGY: From the Greek μακρος, large, and φυσα, bladder, in reference to the very large inflated vesicle on the ventral, apical side of the ovary, much larger than is normal for this group of species.



Authors: E. Hágsater, O. Pérez & E. Santiago **Illustrator: O. Pérez** **Photo: O. Pérez** **Editors: E. Hágsater & L. Sánchez S.**

Herbario AMO **México, D.F. MÉXICO** **ICONES ORCHIDACEARUM 14. 2013.** **Plate 1453**



EPIDENDRUM PACHYCOLEUM Hágsater, O.Pérez & E.Santiago **Plate 1466**

EPIDENDRUM PACHYCOLEUM Hágsater, O.Pérez *et* E.Santiago, *sp. nov.*

Type: COLOMBIA: Valle del Cauca; Municipio el Cerrito; Tenerife, Páramo "Pan de Azúcar" [Páramo Las Hermosas], 3600 m, 6 January 2011, ***Oscar Alejandro Pérez Escobar & Marta Kolanowska 873.*** Holotype: CUVC! (Illustration voucher), digital images of pretype, AMO!

Similar to *Epidendrum serpens* Lindl. but the pseudobulb 1-2-leaved, leaves elliptic, acute, and a single reddish-violet flower.

Epiphytic, sympodial, rhizomatous **herb** *ca.* 5 cm tall. **Roots** 1-2 mm thick, basal, thin, fleshy, scarce, green with white and burgundy-red tinges. **Stems** 0.45-1 x 0.6-0.7 cm, aggregate, thickened, forming globose, homoblastic, pseudobulbs; covered by 1-3 bracts 11-14 x 8-10 mm, imbricated, unequal in size, papiraceous, the veins prominent. **Leaves** 1-2, apical, leaf apparently not articulate to the very fleshy, appresed sheath which appears to be part of the pseudobulb, coriaceous, dark green tinged violet towards the margins and underside, the juvenile leaves burgundy-red; blade 1.0-2.5 x 0.5-1.0 cm, elliptic, acute, margin hyaline, spreading, erose. **Spathaceous bract** lacking. **Inflorescence** apical, 2-3-flowered, sessile, rachis very short and thick. **Floral bracts** ca. 2 mm long, very small, triangular, obtuse. **Ovary** 6 mm long, terete, not inflated, unornamented. **Flower** 2-3, flowers developing in succession, with 2 sometimes open at one time, resupinate, reddish violet, the column and the disc of the lip yellowish red; fragrance not registered. **Sepals** spreading, free, ovate, acute, fleshy, 3-veined, margin entire, spreading; dorsal sepal 7 x 3 mm, lateral sepals 7.3 x 4 mm, oblique, dorsally pustulate, with an apical low, dorsal keel. **Petals** *ca.* 7 x 2.1 mm, free, spreading, narrowly oblong, acute, 1-2-veined, margin entire, spreading. **Lip** 5.5 x 8 mm, united to the column, widely cordiform, apiculate, slightly concave in natural position, margin irregularly dentate; ecallose, with a wide, low, central, prominent rib, elongated to the apicule. **Column** 3.5 mm long, short, thick, straight, forming a right angle with the ovary. **Clinandrium-hood** reduced, entire. **Anther** not seen. **Pollinia** 4, obovoid, sub-equal; caudicles soft and granulose, slightly longer than the pollinia, wide; viscidium semi-liquid. **Rostellum** sub-apical, slit. **Lateral lobes of the stigma** not seen. **Nectary** not seen. **Capsule** not seen

OTHER SPECIMENS: COLOMBIA: Valle del Cauca: Mpio: Tuluá: Alto de Barragán, Cañón Garrapatas, ca. 3300 m, 27 IV 2012, *E. Parra 912*, digital image series, AMO!

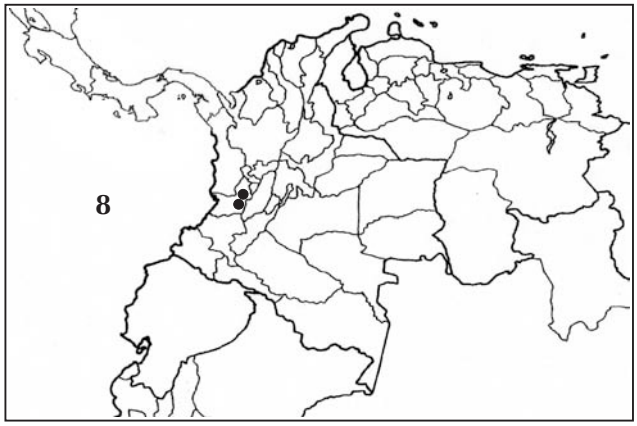
DISTRIBUTION AND ECOLOGY: Known from two localities in the Valle del Cauca, Colombia: near the summit of the Cordillera Central, at 3300-3600 m altitude. Flowering in January. It grows epiphyitically on small, isolated shrubs found reaching the paramo, beside the road.

RECOGNITION: *Epidendrum pachycoleum* belongs to the Kalopternix Group, which has single to few reddish-brown to purple flowers, often resupinate, the petals narrower than the sepals, lip more or less cordiform, sometimes apically 3-lobed, ecallose, usually with a thickened, low, rounded rib running down the middle, the column short, forming a right angle with the ovary, which is short, and the Serpens Subgroup which has plants with aggregate, globose pseudobulbs with fleshy-coriaceous leaves, and a sessile inflorescence, and one or few fleshy, compact, star-shaped flowers, often burgundy red in color, lip entire, more or less cordiform. The species is recognized by the erect plant, 1-2-leaved, aggregate, homoblastic pseudobulbs, elliptic leaves, the apical one often much reduced, sessile flowers, produced in succession, sometimes 2 open at one time, sepals 7.0-7.3 mm long. *Epidendrum serpens* Lindl. also has an erect plant, but 2-3 leaves per pseudobulb, 4-5 simultaneous flowers, leaves ovate-lanceolate, linear-lanceolate petals, the lip sub-rounded–ovate. *Epidendrum platyphylloserpens* Hágsater, from Ecuador, has pendent leaf, oblong-elliptic, acute, with up to 9 flowers opening in succession, several open at one time, the sepals are 5-veined, 8.5 mm long. *Epidendrum pachacuteqianum* Hágsater & Collantes, from Peru, has a single erect leaf per stem, small, successive, non-resupinate flowers, sepals 6-8 mm long, the margin of the lip and petals minutely papillose.

CONSERVATION STATUS: DD. Data deficient.

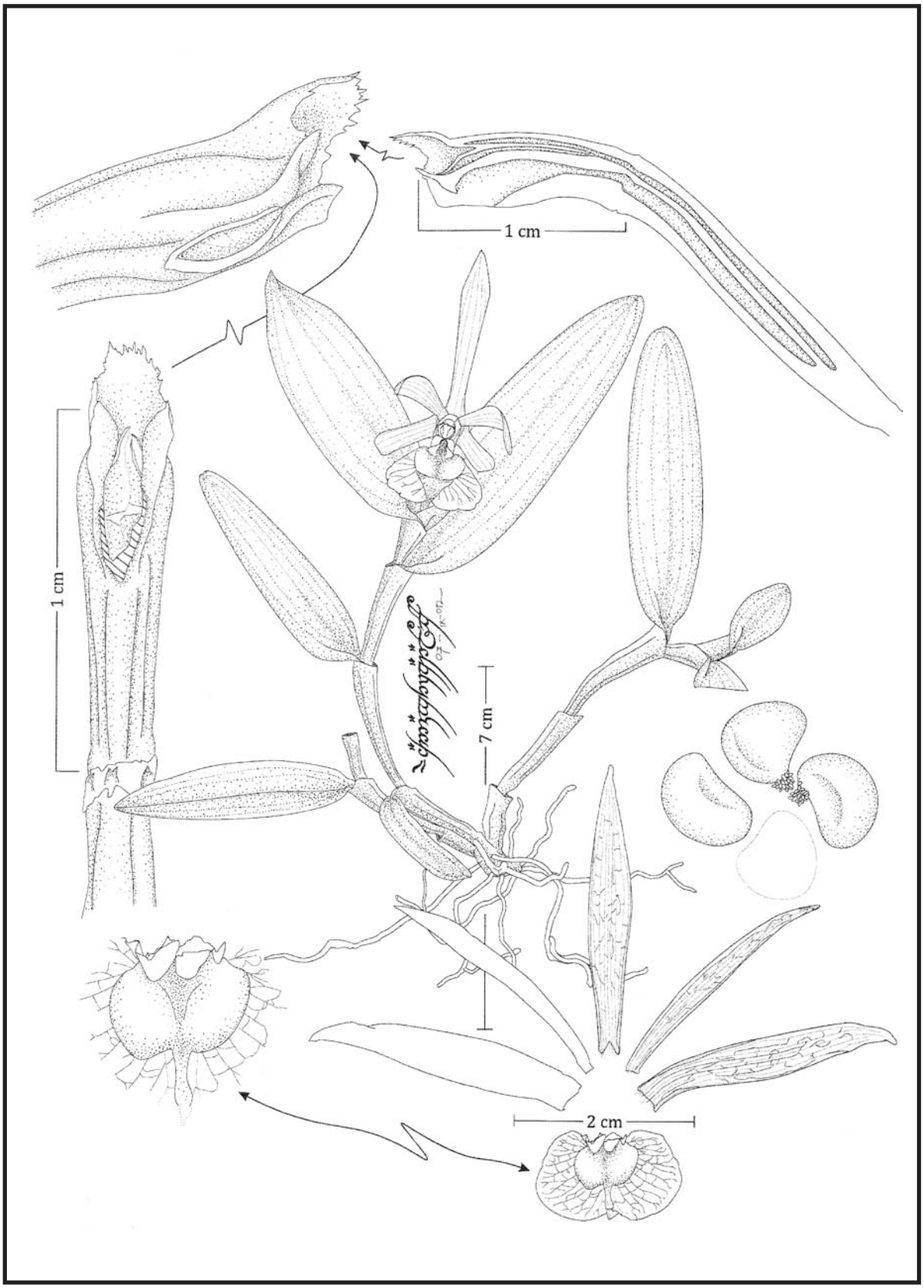
ETYMOLOGY: From the Greek παχυς, thick, κολεος, vagina, in reference to the thickened, fleshy sheath of the lower leaf which envelops the pseudobulb.

REFERENCES: Hágsater, E., 2001, *Epidendrum platyphylloserpens*, in The Genus Epidendrum, **Icon. Orchid.** 4: pl. 473. Hágsater, E., & B. Collantes, 2006, *Epidendrum pachuteqianum*, in The Genus Epidendrum, **Icon. Orchid.** 8: pl. 864.



Authors: E. Hágsater, O. Pérez & E. Santiago **Illustrator: O. Pérez** **Photo: O. Pérez** **Editors: E. Hágsater & L. Sánchez S.**

Herbario AMO **México, D.F. MÉXICO** **ICONES ORCHIDACEARUM 14. 2013.** **Plate 1466**



EPIDENDRUM PARRA-SANCHEZII Hágsater, O.Pérez & L.Sánchez **Plate 1471**

EPIDENDRUM PARRA-SANCHEZII Hágsater, O.Pérez *et* L.Sánchez, *sp. nov.*

Type: COLOMBIA: Valle del Cauca: municipio de Buenaventura, San Cipriano, aprox. 100 m.s.n.m., 30°C, 20 III 2010, ***Oscar Alejandro Pérez Escobar, Edicson Parra Sánchez, Carlos Jaramillo & Paola Narváez 631.*** Holotype: CUVC! (Illustration voucher).

Similar to *Epidendrum sympetalostele* Hágsater & L.Sánchez, the petals free, lip bilobed, reniform, apical margin sinuate, disc deep green, with two lateral ridges which delimit it and form a fleshy, lustrous, shallow cavity, column apex with a small, acute tooth on each side.

Epiphytic, erect, sympodial, caespitose **herb** ca. 12 cm tall. **Roots** 0.5-1.0 cm in diameter, basal, fleshy, filiform. **Stems** 5.0-6.5 x 0.4-0.5 cm, simple, cane-like, laterally compressed towards the apex, flexuous. **Leaves** 3-4, distributed throughout the stem; sheath 2.0-2.2 cm long, tubular, laterally compressed, smooth; blade 2-7 x 0.5-1.9 cm, elliptic to lanceolate-elliptic, apex retuse to asymmetrically bilobed, minutely aristate, coriaceous, green, the primary veins marked pale green on the dorsal surface, margin entire, spreading, pale green. **Spathaceous bract** lacking. **Inflorescence** apical, 1-2-flowered, sessile. **Flowers** 2, simultaneous, resupinate, sepals, petals, lip and proximal and middle part of the column pale green; disc, calli and distal part of the column deep green; fragrance not registered. **Ovary** 21.0 x 2.6 mm, terete, inflated, unornamented. **Sepals** spreading, free, acute, membranaceous, 7-8-veined, with many short, interconnecting secondary veins, margin entire, revolute; **dorsal** sepal 32.0 x 5.5 mm, erect, narrowly elliptic; **lateral** sepals 30-31 x 5.2-6.0 mm, reflexed, oblanceolate, slightly constricted towards the base, obscurely falcate. **Petals** 28-29 x 3 mm, partly spreading, linear-lanceolate, obscurely falcate, acute, membranaceous, 3-veined, margin entire. **Lip** 10 x 16 mm, united to the column, bilobed, strongly convex in natural position, reniform, base cordate, apex sinuate, when flattened the apical margin of the lip will overlap, so it may thus appear apiculate, perpendicular to the axis of the column, margin entire; bicallose, calli small, sub-globose; disc deep green, with two lateral ridges which delimit it and form a fleshy, lustrous, shallow cavity. **Column** 11.2 mm long, slightly arched with a small, acute tooth in each side of the apex. **Clinandrium-hood** prominent, irregularly dentate. **Anther** obovoid, apex obtuse; 4-celled. **Pollinia** 4, reniform, laterally compressed, caudicles granulose, very short. **Rostellum** sub-apical, slit. **Lateral lobes of the stigma** small, covering 1/2 of the stigmatic cavity. **Nectary** penetrating 2/3 of the ovary, unornamented. **Capsule** not seen.

OTHER SPECIMENS: COLOMBIA: Valle del Cauca: Municipio de Buenaventura, La Delfina, aprox. 100 m, 30 III 2007, *Pérez 468,* CUVC! Ibid. 4 I 2011, *Kolanowska 269,* UGDA! Digital images of live plant taken by Marta Kolanowska, AMO! (Photo voucher.)

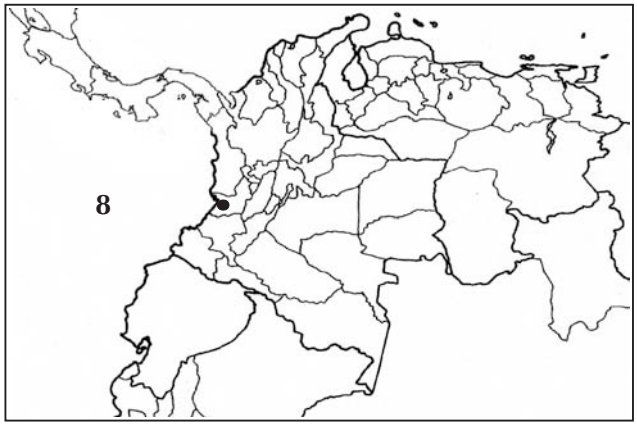
DISTRIBUTION AND ECOLOGY: Endemic to the Chocó biogeographic region and known only from the Buenaventura municipality on the department of Valle del Cauca, epiphytic, at low elevations (ca. 100 m), on isolated, mature, trees in disturbed places. Individuals and small populations have been seen growing on mature trees of *Jacaranda* sp. (probably *J. caucana* Pittier) and *Inga* sp. Flowering January-March.

RECOGNITION: *Epidendrum parra-sanchezii* belongs to the Difforme Group which is characterized by the caespitose, sympodial plants, fleshy pale green to glaucous leaves, apical inflorescence without the spathaceous bract, sessile, rarely with a short peduncle, one-flowered to corymbose, fleshy, and flowers green to yellowish-green, rarely white. The species is recognized by the its small plants (ca. 12 cm tall), stems laterally compressed, with 3-4 elliptic to lanceolate-elliptic leaves, inflorescences 1-2-flowered, a bilobed, reniform, convex lip, disc bordered by two ridges, which delimit it, forming a fleshy and lustrous, deep green, shallow cavity. *Epidendrum sympetalostele* is vegetatively similar, but differs mainly by the sub-erect petals adnate to the basal half of the column, disc without ridges on the side, and prominent bifid lobes at the sides of the apex of the column. *Epidendrum kerryae* Hágsater & L.Sánchez has a single, large flower, trigonous ovary with a ventral vesicle which is dorsally flat, and an erose clinandrium-hood, the lip is entire, sub-orbicular, disc unornamented. *Epidendrum putumayoënse* Hágsater & L.Sánchez from the Amazonas slope of the southern of Colombia and northern Ecuador, has 1-flowered inflorescences, a 3-lobed, obtrapezoid lip, disc wrinkled at the base with three low ribs, the central one prominent, extended until the apex, the lateral ones reaching the middle of the lip, column straight, truncate, clinandrium-hood irregularly toothed.

CONSERVATION STATUS: DD. Data deficient.

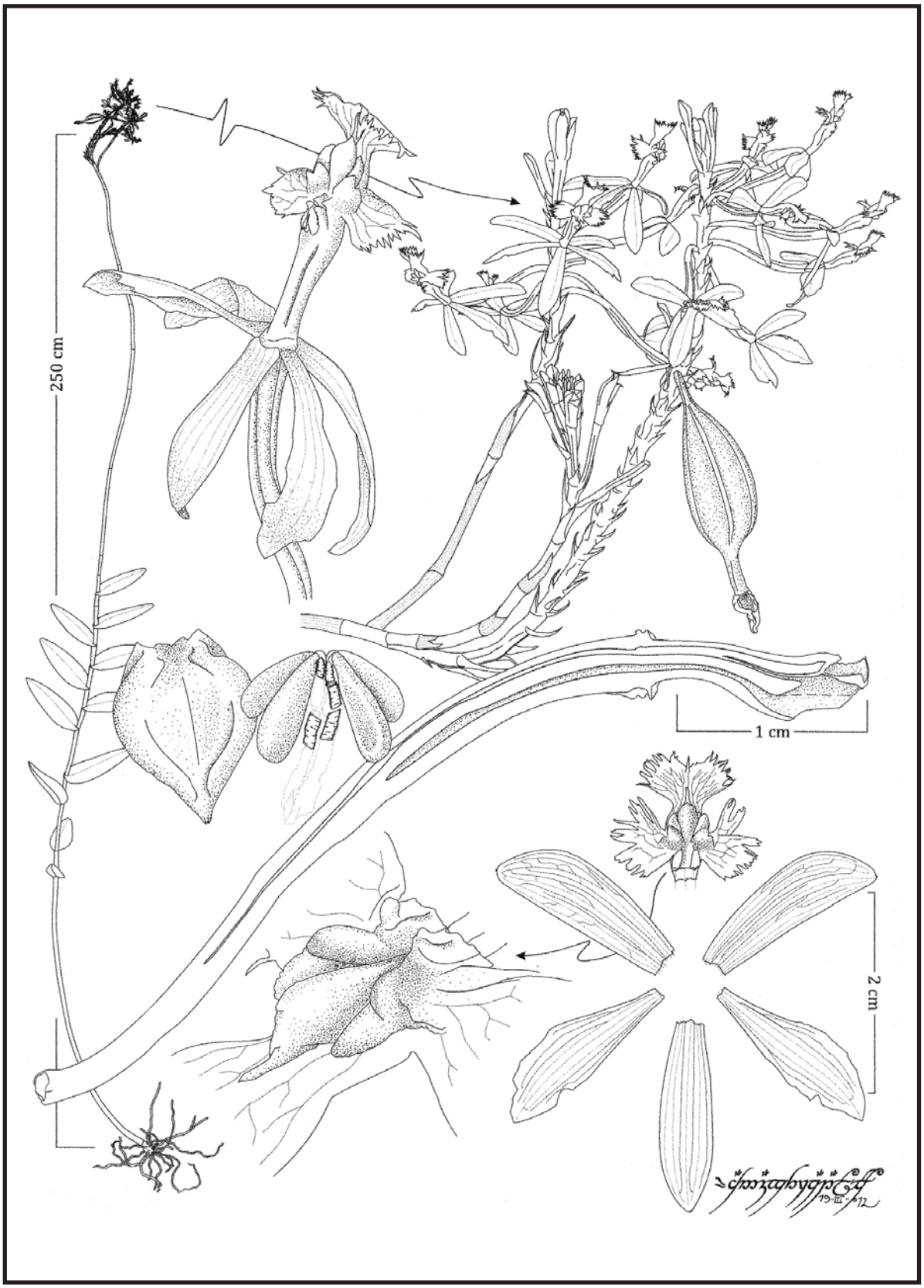
ETYMOLOGY: In honor of Edicson Parra-Sánchez, agronomy engineer graduate from the Universidad Nacional de Colombia, and colleague and friend of the second author. He is an enthusiastic student of orchid taxonomy and has contributed with his work and dedication to the knowledge of the Orchid Flora of the department of Valle del Cauca, Colombia.

REFERENCES: Hágsater, E. & L. M. Sánchez Saldaña, 1994, *Epidendrum kerryae*, una nueva especie de Colombia, **Orquideología** 19(2): 37-42. Hágsater, E. & L. Sánchez Saldaña, 1993, *Epidendrum sympetalostele*, in E. Hágsater & G. A. Salazar (eds.) **Icon. Orchid.** 2: pl. 191. Hágsater, E. & L. Sánchez Saldaña, 1999, *Epidendrum putumayoënse*, in Hágsater, E., L. Sánchez Saldaña & J. García-Cruz (eds.) **Icon. Orchid.** 3: pl. 377.



Authors: E. Hágsater, O. Pérez & L. Sánchez S. Illustrator: O. Pérez Photo: M. Kolanowska Editors: E. Hágsater & L. Sánchez S.

Herbario AMO México, D.F. MÉXICO ICONES ORCHIDACEARUM 14. 2013. Plate 1471



EPIDENDRUM SUSANNAE Hágsater, O.Pérez & E.Parra **Plate 1486**

EPIDENDRUM SUSANNAE Hágsater, O.Pérez *et* E.Parra *sp. nov.*

Type: COLOMBIA: Boyacá: Municipio de Arcabuco; Alto de Gaitas, ca. Reserva "Rogitama", ca. 2600 m, aprox. 7° C, 3 October 2011, ***Oscar Alejandro Pérez Escobar & Edicson Parra Sánchez 1105.*** Holotype: VALLE! (Illustration voucher).

Simile *Epidendri reflexilobi* C.Schweinf. sed floribus maioribus colore roseo-magenteo, labello pallide lilacino, callis albis et callis apicalibus prominentibus, praesertim centrali.

Terrestrial, caespitose, erect **herb**, to 265 cm tall. **Roots** 1.3-2.3 mm in diameter, produced from the base of the stems, fleshy, thick. **Stems** simple, cane-like, 143 x 0.9-1.18 [1.3] cm, terete, thick, straight. **Leaves** 8-15, articulate, distributed along the upper half of the stem; sheaths tubular, 3.12-6.7 x 1.0-1.4 cm, smooth to striated, green tinged with dark purple; blade 9.2-16 x 2.9-4.3 cm, lanceolate, coriaceous, apex obtuse, rounded, short bilobed, margin entire. **Spathaceous bract** lacking. **Inflorescence** 122 cm long, apical, erect, pluri-racemose; peduncle 87.6 cm long, elongate, terete, thin, generally covered by amplexicaul bracts; each many-flowered raceme 8.6-19.8 cm long, compact, dense, its peduncle covered by 5 tubular bracts 1.9-3.6 cm long, acute, scarious, becoming fibrous with time. **Flowers** numerous, 19-45 per raceme, successive, 5-6 open at one time, non-resupinate, rose-magenta, the lip pale lilac with the throat orange-yellow, the calli and margin of the clinandrium white. **Floral bracts** 3-13.8 mm long, much shorter than the ovary, triangular, acuminate, gradually shorter towards the apex of the rachis. **Ovary** 21.4-40 mm long, thin, not inflated, striated, angulate towards the base. **Sepals** 18-19.1 x 5-6.1 mm, spreading to slightly recurved, free, oblong-elliptic, acute, minutely apiculate, 7-veined, the veins branched so as to appear 8-9 veined, margin entire, spreading; lateral sepals oblique. **Petals** 19.5-20 x 6-6.1 mm, spreading to slightly recurved, free, oblanceolate, slightly oblique, acute, 5-veined, the lateral veins branched, so as to appear 8-veined, apical half of the margin erose, spreading. **Lip** 13 x 15 mm, united to the column, 3-lobed, base cordate, apical margin of the lobes fimbriate; callus massive, formed by two, small, basal, bilobed calli, followed by 3 calli, the central one large, smooth, terminating in a short, thin rib that disappears before the apical sinus, the lateral pair smaller; lateral lobes 6 x 8 mm, dolabriform, the forward margin conduplicate in natural position; mid-lobe 8 x 10 mm, with a short, narrow isthmus at the base, then bilobed, emarginate, with a small mucro in the sinus, the lobes flabellate, divaricate. **Column** 9-12 mm long, short, straight, thin at the base, and gradually thicker towards the apical half. **Clinandrium-hood** reduced, margin dentate. **Anther** ovoid, acute, 4-celled. **Pollinia** 4, obovoid, laterally slightly compressed, similar in size, caudicles twice as long as the pollinia, formed by imbricated tetrads, appearing as a pile of tiles. **Rostellum** apical, slit. **Lateral lobes of the stigma** not seen. **Nectary** penetrating 1/3 of the ovary, thin, not inflated, papillose. **Capsule** ovoid, green with the valves tinged pale purple.

OTHER SPECIMENS: None seen.

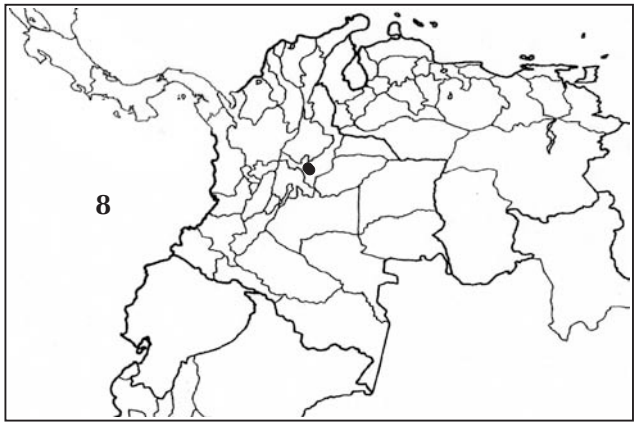
OTHER RECORDS: COLOMBIA: Boyacá: Mpio. Arcabuco; Alto de Gaitas, ca. Reserva "Rogitama", 2600 m, 3 X 2011, *Pérez s.n.* Digital images, AMO! (Image voucher).

DISTRIBUTION AND ECOLOGY: So far known only from the Cordillera Oriental of the Andes, in Boyacá Department, Arcabuco municipality, at 2600 m. Just two individuals are reported from the type locality, growing as terrestrial at the border of a fragmented forest.

RECOGNITION: *Epidendrum susannae* belongs to the Secundum Group which is recognized by the caespitose habit, numerous coriaceous leaves, and generally an elongate peduncle to a pluri-racemose inflorescence, brightly colored flowers generally pollinated by hummingbirds, and the caudicles of the pollinarium granulose, the tetrads appearing like a loose pile of roof-tiles, without any spathaceous bracts; and Elongatum Sub-group, recognized by the non-resupinate flowers with a complicated callus. This species is terrestrial and has bright purple flowers, the lip pale lilac, with the throat at the base of the lip orange-yellow, and the calli white, formed by two basal, lateral calli, and the main body by a large central entire tubercle, embraced by a pair of shorter lateral tubercles. *Epidendrum reflexilobum* C.Schweinf. from Huánuco, Peru, has scarlet flowers with the disc of the lip yellow, sepals 12-13 mm long, disc with 3 short fleshy keels, the central one longest and the lateral ones with the fleshy lobulated base spread onto the lateral lobes of the lip. *Epidendrum arachnoglossum* André, a common species around Bogotá, has violet-crimson flowers, a many-lobed white and orange-yellow callus, and the deeply fringed lip forms a nearly entire, orbicular lamina, the base cordate.

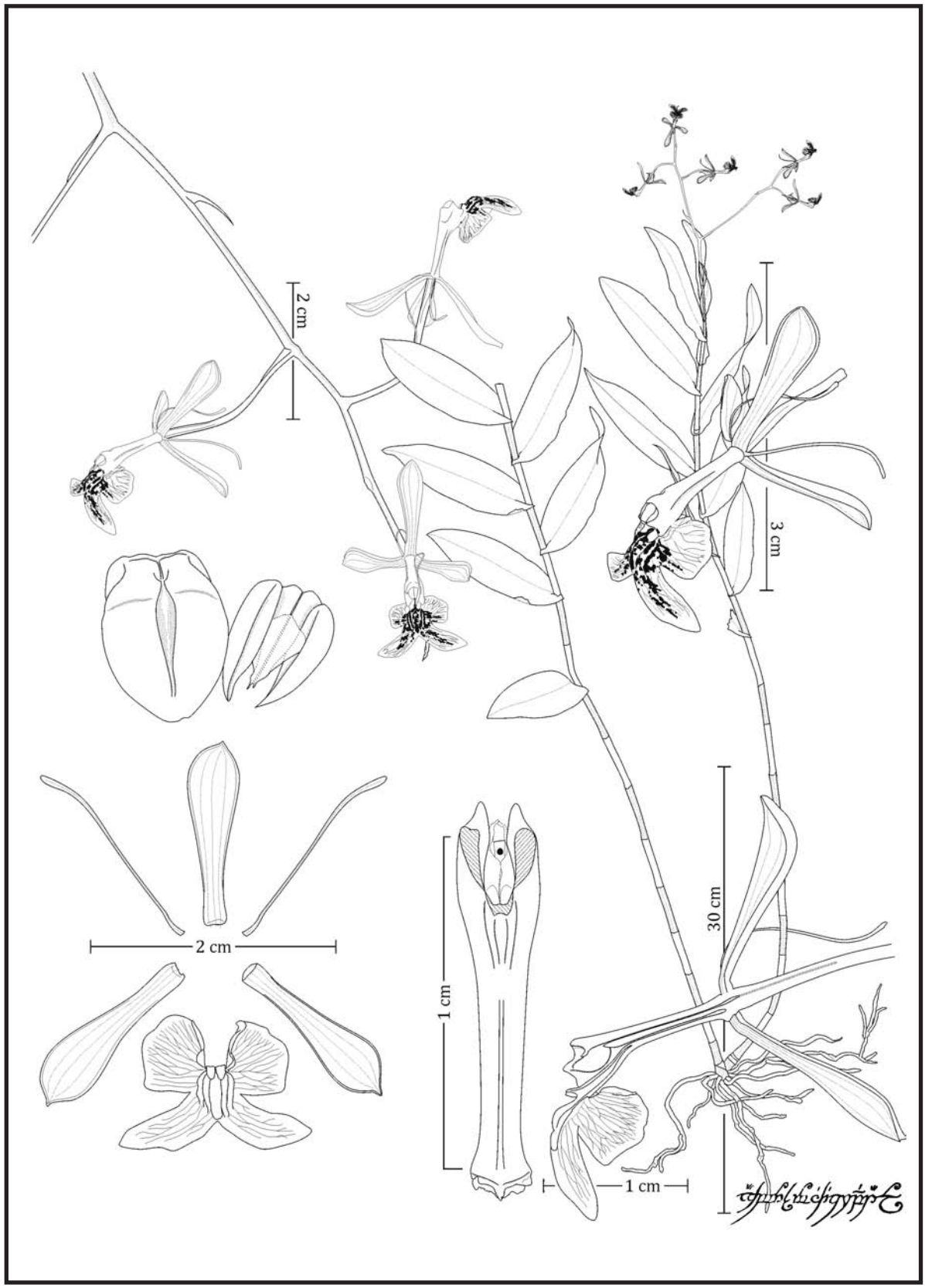
CONSERVATION STATUS: D.D. Data deficient

ETYMOLOGY: in honor of Dr. Susanne S. Renner (Germany, 1954), Professor at the Ludwig-Maximilians Universität, Chief Director of the Botanische Garten München and mentor of the second author, in recognition to her important contributions to the knowledge on phylogeny and biogeography of Cucurbitaceae, Melastomataceae and several other monocot families, as well as in the field of evolution of reproductive systems.



Authors: E. Hágsater, O. Pérez & E. Parra Illustrators: O. Pérez & R. Jiménez M. Photo: O. Pérez Editors: E. Hágsater & L. Sánchez S.

Herbario AMO México, D.F. MÉXICO ICONES ORCHIDACEARUM 14. 2013. Plate 1486



EPIDENDRUM WEERAKITIANUM Hágsater, O.Pérez & E.Santiago **Plate 1495**

EPIDENDRUM WEERAKITIANUM Hágsater, O.Pérez *et* E.Santiago, *sp. nov.*

Type: COLOMBIA: Valle del Cauca: Municipio de Yumbo; Dapa, 3 April 2011, ***Oscar Alejandro Pérez Escobar & Edicson Parra Sánchez 1106***. Holotype: VALLE! (Illustration voucher).

Simile *Epidendri paniculati* Ruiz & Pav. sed floribus paulo maioribus, lobulis lateralibus labelli dolabriformibus y disco praedito 5 costis fortiter purpureo signatis, basi lobulorum labelli similiter purpureo signatis.

Lithophytic, rupicolous, sympodial, caespitose, decumbent **herb**, ca. 65 cm tall. **Roots** ca. 3 mm in diameter, basal, fleshy, thin. **Stems** simple, cane-like, terete, 50 x 0.6 cm; the basal half covered by non-foliar, minutely striated, tubular sheaths 1.5-3.6 cm long. **Leaves** 12, distributed throughout the apical half of the stem, alternate, articulate, amplexicaul, erect-spreading, similar in size; sheath tubular, 1.2-3.0 x 0.3-0.6 cm, minutely striated, green; blade elliptic, 7-11 x 2-3.3 cm, acute, sub-coriaceous, margin entire, spreading. **Spathaceous bract** lacking. **Inflorescence** apical, 14 cm long, paniculate, flowering only once, lax-, few-flowered; peduncle short, 5 cm long, straight, thin, provided with 1 lanceolate, acuminate, amplexicaul bract 1.5 cm long; rachis 9 cm long. **Floral bracts** 6-8 mm long, much shorter than the ovary, narrowly triangular, acuminate, amplexicaul. **Ovary** 21-22 mm long, terete, thin, not inflated, arching at the apical 1/3. **Flowers** 10-20, simultaneous, resupinate, sepals, petals and basal half of the column green, apical half of the column and lip white (turning yellowish with time), the lip with 5 reddish-purple lines on the ribs, lobes of the lip densely spotted with reddish-purple: fragrance not recorded. **Sepals** 15 x 4.3 mm, reflexed, free, oblanceolate-spatulate, obtuse, fleshy, slightly concave towards the apex, 3-veined, margin entire, spreading. **Petals** 17 x 0.7 mm, reflexed, free, filiform, apex rounded, 1-veined, oblique, margin entire, spreading. **Lip** united to the column, 10 x 15 mm, slightly convex, fleshy, 3-lobed, base cordate, margin entire; bicallose, the calli prominent, rectangular, disc provided with 5 , fleshy, parallel ribs, which extend nearly to the apical sinus; lateral lobes dolabriform, 4.3 x 6 mm; mid-lobe 4.7 x 15 mm, widely emarginate, forming a pair of linear, acute, slightly divaricate lobes, each lobe 7.2 x 3.2 mm. **Column** 10 mm long, straight, thin along the basal 2/3, and gradually dilated towards the apex. **Clinandrium-hood** reduced, margin entire. **Anther** ovoid, 4-celled, with a low dorsal keel. **Pollinia** 4, bird-wing type; caudicles laminar, somewhat shorter than the pollinia. **Rostellum** apical, slit. **Lateral lobes of the stigma** prominent. **Nectary** thin, unornamented, without penetrating the ovary. **Capsule** not seen.

OTHER SPECIMENS: COLOMBIA: Valle del Cauca: Represa del Calima, 17 IX 1966, *Espinal 2041*, MO!

OTHER RECORDS: COLOMBIA: Without locality data, as *E.* aff *paniculatum*(1 y 2) photo *C. Uribe s.n.* (Ortiz & Uribe, 2007). Ibid. as *E. rodrigoi 2* photos *C. Uribe s.n.,* (Ortiz & Uribe, 2007). **Antioquia:** without locality data, *G. Escobar 677*, slide, AMO! **Quindio:** Circasia, without collector data, photo published as *Epidendrum paniculatum* (Mejía de Moreno, 2007). **Valle del Cauca:** localidad Yumbo; corregimiento de Dapa, 1800 m, 13 XII 2009, *Parra & Pérez s.n.*, digital image, AMO!

DISTRIBUTION AND ECOLOGY: Known only from the Cordillera Occidental in southern Colombia the Cordillera Central in Quindío and Antioquia, lithophytic at 1770-2050 m altitude. Flowering from September to December, April.

RECOGNITION: *Epidendrum weerakitianum* belongs to the Pseudepidendrum Group which is characterized by caespitose plants, cane-like stems, acute to acuminate leaves, usually apical inflorescence, the mostly filiform petals and the lip usually 3-lobed (with 3 parallel fleshy keels), the apical lobe often bifurcate, the "bird-wing" type pollinia, at least the inner pair, and Paniculatum Subgroup, which has filiform petals, all pollinia "bird-wing" type, green and white flowers, often marked with purple on the disc of the lip and apex of the column. The species is recognized by the mid-sized plants (65 cm tall), elliptic leaves, lax, few-flowered inflorescences, green colored flowers with the apex of the column and lip white, the lip with 5 ribs stained with purple, the reddish-purple spots spilled out towards the base of the lateral lobes. *Epidendrum paniculorotundifolium* Hágsater, M.Kolanowska & E.Santiago has similar plants, but with elliptical-orbicular leaves, green colored flowers with the column and lip white, disc of the lip with three ribs, immaculate. *Epidedrum kolanowskae* Hágsater, O.Pérez *et* E.Santiago has elliptic leaves, green colored flowers, with the apex of the column and lip white, the lip with three ribs and the disc stained of purple pale around the ribs of the disc and incipiently spilled out over the lobes of the lip. *Epidendrum paniculatum* Ruiz & Pav. has smaller flowers, (sepals 10-12 mm long), the lateral lobes of the lip sub–orbicular, and the mid-lobe formed by two linear-oblong, falcate, strongly divaricate lobes, the 3-ribbed disc is immaculate and surrounded by reddish-purple marks.

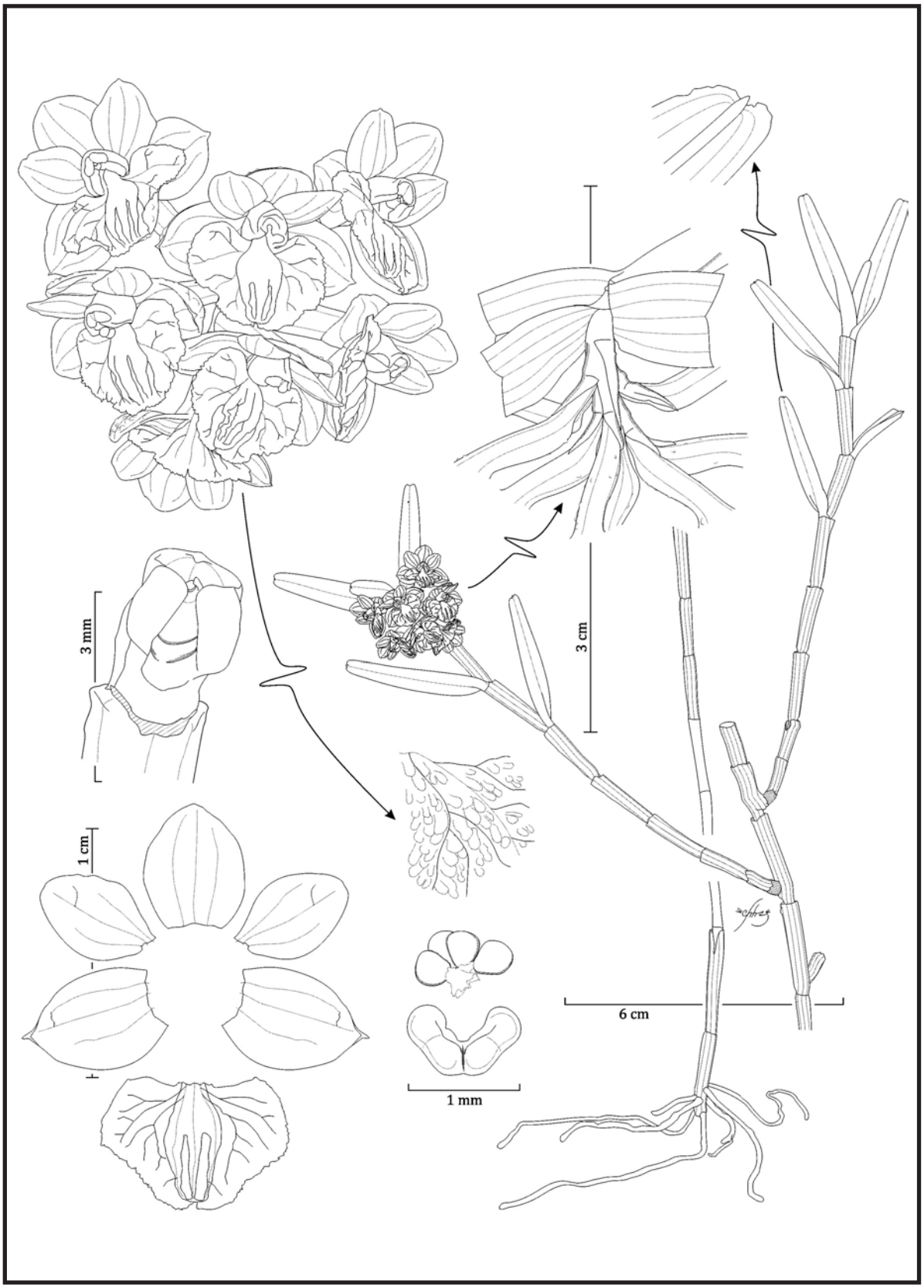
CONSERVATION STATUS: DD. Data deficient.

ETYMOLOGY: In honor of Weerakit Harnpariphan (1955-), a medical doctor from Bangkok (Thailand), who has contributed greatly to the conservation of Colombian flora, especially of native species of Magnoliaceae from this country.

REFERENCES: Mejía de Moreno, E. 2009. ***Orquídeas del Quindío***, Litografía Luz Armenia, pág. 58, Colombia. Ortiz V., P. & C. Uribe V. 2007, ***Gallery of Colombian Orchids***, Da Vinci Editores, Bogotá-Colombia (DVD).

Authors: E. Hágsater, O. Pérez & E. Santiago Illustrator: O. Pérez Photos: G. Escobar Editors: E. Hágsater & L. Sánchez S.

Herbario AMO México, D.F. MÉXICO ICONES ORCHIDACEARUM 14. 2013. Plate 1495



EPIDENDRUM YUMBOËNSE Hágsater, O.Pérez & E.Santiago **Plate 1499**

EPIDENDRUM YUMBOËNSE Hágsater, O.Pérez *et* E.Santiago, *sp. nov.*

Type: COLOMBIA: Valle del Cauca: Municipio de Yumbo; Dapa, growing on trees of *Meriania* and *Tibouchina* (Melastomataceae), 2000 m, 20° C, 28 August 2010, ***Oscar Alejandro Pérez Escobar, Terry González & Ángela González 831.*** Holotype: VALLE! (Illustration voucher). Isotype: CUVC! (Digital images of pretype, AMO! photo voucher.)

Simile *Epidendri podocarpophili* Schltr. sed floribus pallide roseis disco aurantiaco, petalis ovatis marginibus integris et labello late reniformi-pentagonali praedito 3 carinis singularibus, brevibus atque tenuibus coalescentibus in unam carinam versus apicem laminae.

Epiphytic, monopodial, branching **herb** 28-41 cm tall**. Roots** basal, both from the basal stem as well as occasionally from branches, fleshy, white. **Stems** cane-like, terete, thin, erect, straight; main stem 20 x 0.3 cm; branching when mature, the branches 10 x 0.3 cm, arising from the sub-apical internodes of the previous stem. **Leaves** 6-7, distributed along the apical half of the stems, erect-spreading, alternate, articulate, coriaceous, green with the margin tinged purple; sheaths 3-20 x 3-4 mm, tubular, striated, purple-green; blade 11-40 x 3.7-6.5 mm, oblong-lanceolate, apex truncate, bilobed, minutely aristate, margin entire. **Spthaceous bract** lacking. **Inflorescence** apical, racemose, arching-nutant, short, dense-flowered, peduncle ca. 7 mm long, rachis very short. **Floral bracts** 2-5 mm long, much shorter than the ovary, triangular-lanceolate, acuminate. **Flowers** 9, simultaneous, resupinate, small, fleshy, glabrous, pustulate at the upper side of the elements of the perianth, pale pink to yellow with the disc orange-yellow; fragrance not registered. **Ovary** 8.5 mm long, slightly flattened, ventrally inflated along the apical 2/3, forming an obvious elongate vesicle, scarcely pustulate, arched towards the apex. **Sepals** spreading, free, slightly concave, 3-veined, margin entire, spreading; **dorsal sepal** 5 x 4 mm, ovate-orbicular, apex rounded; the **lateral sepals** 6 x 4 mm, obovate, sub-obtuse, apex mucronate. **Petals** 5 x 3.1 mm, spreading, free, ovate, wide, apex rounded, base oblique, unequal, 3-veined, margin entire, spreading. **Lip** 5.5 x 7.5 mm, united to the basal half of the column, widely reniform-pentagonal, base cordate, apex shallow-emarginate, margin irregularly erose-denticulate, entire towards the base; ecallose, disc with 3 smooth ribs extending to the apex of the lip, and fused towards the base into one wide thickening. **Column** 3 mm long, short, thick, slightly arched with respect to the ovary, straight. **Clinandrium-hood** short, margin entire. **Anther** reniform, 4-celled. **Pollinia** 4, obovate, laterally compressed, hard. **Rostellum** apical, slit. **Lateral lobes of the stigma** not seen. **Nectary** deep, penetrating ¾ of the ovary, wide, unornamented. **Capsule** note seen.

OTHER SPECIMENS: COLOMBIA: Valle del Cauca: Cordillère Occidentale de Cali, 2000 m, 15 VIII 1883, *Lehmann 3022*, G! Yumbo, Dapa, en frente de la Finca "Cielo Azul", ca. 1900 m, 31 VII 2010, *Pérez 818*, CUVC! Cerro El Ingles, Serranía de los Paraguas, 2260-2300 m, 3 I 1987, *Silverstone-Sopkin 2903*, AMO! CUVC! MO!

OTHER RECORDS: COLOMBIA: Valle del Cauca: Mun. El Cairo, Reserva Natural Cerro El Ingles, 2169 m, 12 VII 2011, *García-Revelo 13*, digital image, AMO!

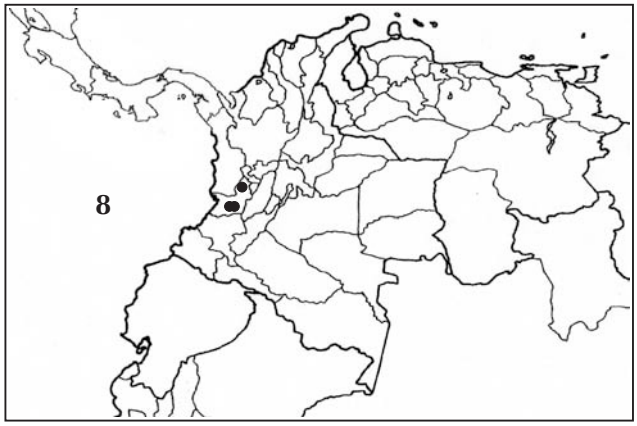
DISTRIBUTION AND ECOLOGY: So far only known from the upper Pacific slope of the western Cordillera of the Andes, in southern Colombia, in the Valle del Cauca. Epiphytic at 2000-2300 m in cloud forest; grows frequently on trees of *Meriania sp.* and *Tibouchina sp.* (Melastomataceae), at the edge of forest. Flowering from July to January.

RECOGNITION: *Epidendrum yumboëns*e belongs to the Diothonea Group and Subgroup, characterized by the branching habit, linear-lanceolate to lanceolate, bilobed leaves, arching-nutant, racemose inflorescence, membranaceous or rarely fleshy flowers, the lip entire to 3-lobed, with an erose margin, ecallose, without or with 1-10 thin, smooth to erose keels, the column united to the lip from totally to obliquely to free. The species is recognized by the oblong-lanceolate leaves 1.1-4 cm long, ovary ventrally inflated along the apical 2/3, forming an obvious elongate vesicle, sparsely pustulate, inflorescence with some 9, pale pink to yellow flowers, the disc orange-yellow, sepals 5-6 mm long, ovate-orbicular to obovate, oblique, petals ovate, wide, *ca.* 5 x 3 mm, the lip entire, widely reniform-pentagonal, shallowly emarginate with 3 smooth keels, fused at the base, and extending to the apex. *Epidendrum podocarpophilum* Schltr. has pale orange flowers, the ovary is not inflated, oblong-elliptic sepals, obovate-spatulate petals and a 3-lobed lip has 3 short, rounded keels. *Epidendrum caesaris* Hágsater & E.Santiago has oblong-lanceolate leaves 3.8-8.5 cm long, inflorescence with 4-19 pale pink, translucent-colored flowers, ovary slightly inflated, sepals widely elliptical, 8-8.5 mm long, petals narrowly ovate, 7 x 2.9 mm, and a cordiform lip with 3-5 smooth keels than only reach the middle of the lip, and a strongly arched column. *Epidendrum restrepoanum* A.D.Hawkes has carmine-red flowers, the ovary is not inflated, sepals are elliptic-obovate, 6-9 mm long, petals narrowly elliptic, margin slightly erose, 6.8-7.5 mm long.

CONSERVATION STATUS: DD. Data deficient.

ETYMOLOGY: Named after the municipality of Yumbo, Valle del Cauca, where this species has been collected, at higher altitudes in cloud forest.

REFERENCES: Hágsater, E., & E. Santiago, 2007, *Epidendrum caesaris*, in E. Hágsater & L. Sánchez S. (eds.) **Icon. Orchid.** 9: t. 915. Hágsater, E., & E. Santiago, 2007, *Epidendrum restrepoanum*, in E. Hágsater & L. Sánchez S. (eds.) **Icon. Orchid.** 9: t. 979. Santiago, E., & E. Hágsater, 2009, *Epidendrum podocarpophilum,* in E. Hágsater & L. Sánchez S. (eds.) **Icon. Orchid.** 12: t. 1277.



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Chapter 5

RUMBLING ORCHIDS: HOW TO ASSESS DIVERGENT EVOLUTION BETWEEN CHLOROPLAST ENDOSYMBIONTS AND THE NUCLEAR HOST

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Rumbling Orchids: How To Assess Divergent Evolution Between Chloroplast Endosymbionts and the Nuclear Host

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Abstract.—Phylogenetic relationships inferred from multilocus organellar and nuclear DNA data are often difficult to resolve because of evolutionary conflicts among gene trees. However, conflicting or "outlier" associations (i.e., linked pairs of "operational terminal units" in two phylogenies) among these data sets often provide valuable information on evolutionary processes such as chloroplast capture following hybridization, incomplete lineage sorting, and horizontal gene transfer. Statistical tools that to date have been used in cophylogenetic studies only also have the potential to test for the degree of topological congruence between organellar and nuclear data sets and reliably detect outlier associations. Two distance-based methods, namely ParaFit and Procrustean Approach to Cophylogeny (PACo), were used in conjunction to detect those outliers contributing to conflicting phylogenies independently derived from chloroplast and nuclear sequence data. We explored their efficiency of retrieving outlier associations, and the impact of input data (unit branch length and additive trees) between data sets, by using several simulation approaches. To test their performance using real data sets, we additionally inferred the phylogenetic relationships within Neotropical Catasetinae (Epidendroideae, Orchidaceae), which is a suitable group to investigate phylogenetic incongruence because of hybridization processes between some of its constituent species. A comparison between trees derived from chloroplast and nuclear sequence data reflected strong, well-supported incongruence within *Catasetum*, *Cycnoches*, and *Mormodes*. As a result, outliers among chloroplast and nuclear data sets, and in experimental simulations, were successfully detected by PACo when using patristic distance matrices obtained from phylograms, but not from unit branch length trees. The performance of ParaFit was overall inferior compared to PACo, using either phylograms or unit branch lengths as input data. Because workflows for applying cophylogenetic analyses are not standardized yet, we provide a pipeline for executing PACo and ParaFit as well as displaying outlier associations in plots and trees by using the software R. The pipeline renders a method to identify outliers with high reliability and to assess the combinability of the independently derived data sets by means of statistical analyses. [chloroplast capture; cophylogenetic tool; hybridization; orchids; organelle/host nucleus coevolution; topological incongruence.]

INTRODUCTION

Chloroplasts are among the most distinctive organelles and highly specialized compartments in the cells of land plants and algae. Their main role is to perform photosynthesis, converting energy captured from sunlight into chemical bonding of organic substance (Staehelin 2003; Marín-Navarro et al. 2007). Today, it is widely accepted that chloroplasts are of endosymbiotic origin, having evolved from a previously free-living cyanobacterium and hosted by a nucleated, initially heterotrophic cell (the same applies for mitochondria, likely derived from an α-proteobacterium). A plastid genome and distinct plastid ribosomes strongly support an endosymbiotic origin (Mereschkowsky 1910; Margulis 1993; Archibald 2015). The resulting enkaptic and permanent cellular system comprises two principally different genetic units: the eukaryotic host nucleus usually performs recombination during life history, whereas sexual reproduction is known neither from free-living cyanobacteria nor from plastids (Birky 1995; Pyke 1999; Lane 2011; Lodé 2012). Divisions of plastids are structurally independent from the division of the host cell's nucleus (first recognized by Sachs 1882), hence intracellular plastid populations are separated (and frequently intensely cloned) during mitosis in parallel to the host's daughter nuclei (Possingham 1980; Heinhorst and Cannon 1993).

Chloroplast loci have been excessively used for phylogenetic inference because of the great abundance of plastid DNA and the subsequent facility of PCR amplification and sequencing (Rieseberg and Soltis 1991; Schäferhoff et al. 2010; Ruhfel et al. 2014; Weigend et al. 2014). However, the plastid genome has not necessarily tracked the same evolutionary history as the host genome. As a result, the linked but putatively varying evolution of both the (sexually reproducing) nuclear and (solely cloning) plastid genomes may lead to significantly differing substitution rates. Moreover, biological phenomena such as chloroplast capture (e.g., after hybridization or introgression) and incomplete lineage sorting (ILS) of separated plastid populations (Rieseberg and Soltis 1991; Soltis and Kuzoff 1995; Fehrer et al. 2007) may even result in conflicting topologies when molecular trees are inferred separately.

Demonstrating the absence of significant incongruence between any data set partitions is essential for accurate phylogenetic inference (Salichos and Rokas 2013), and this assessment is a general challenge (Wiens and Hollingsworth 2000; van der Niet and Linder 2008). Several comparative methods have been developed to quantify the difference, or the degree of congruence, between two given topologies (e.g.,

partition metrics: Robinson and Foulds 1981; likelihood and Bayesian approaches: Kishino and Hasegawa 1989; Holmes 2005; Charleston 2009). Some others aim at inferring a species tree from a set of genes or entire genomes (e.g., BUCKy: Larget et al. 2010; MP-est: Liu et al. 2010; STEM: Kubatko et al. 2009), irrespectively of the different evolutionary histories reflected by, and despite possible incongruence of, each data partition. The applicability of these approaches is particularly limited for the problem discussed here, because they do not assess the contribution of any given association between the partitions. More importantly, most of these methods are on a "quest for the species tree," and so they neither demonstrate nor explain the existence of phylogenetic incongruence between any two given data sets. Therefore, there is a clear need for a test that can assess not only topological (in)congruence between nuclear and chloroplast data sets, but also the particular associations that contribute significantly to topological incongruence. The recognition of those "outlier" associations (i.e., linked pairs of operational terminal units: OTUs) tracking different phylogenetic histories are doubtlessly of interest (Salichos and Rokas 2013). From an evolutionary perspective, identification of outlier OTUs is even more exciting when it provides useful information on biological events and processes such as horizontal gene transfer (HGT) and ILS (de Vienne et al. 2012).

Most approaches of species tree reconciliation either refer to methodological/computational problems (Ronquist 1995; Page and Charleston 1997; Charleston 1998; Libeskind-Hadas and Charleston 2009; Nakhleh et al. 2009; Larget et al. 2010; Liu et al. 2010) or to biological (intragenomic) phenomena such as gene duplication and loss (Arvestad et al. 2004; Åkerborg et al. 2009), but not to the intergenomic clash investigated in this study. Despite the impact on studies based on multiple molecular loci (Tepe et al. 2011), few approaches have the potential to quantify the contribution of specific taxa to the conflicting phylogenetic patterns observed. We now have at hand a new generation of software applications and tools, which investigate putative cophylogenetic structures in more detail, as they can be observed in, for example, parasite/host systems. They have only been used sporadically so far for specific groups of organisms (e.g., Monogenea/fish: Šimková et al. 2004; papillomaviruses/vertebrates: Gottschling et al. 2011), although they provide a powerful approach to identify those particular associations that are responsible for conflicts.

One of the more frequently employed tools for cophylogenetic analysis has been ParaFit (Legendre et al. 2002), a distance-based approach that globally tests for the coevolution between host and parasite phylogenies and the significance of each parasite/host association. Using patristic/genetic distances transformed into Principal Coordinate (PCo) matrices, it assesses whether the phylogenetic positions of associated taxa in host and parasite trees are congruent (Legendre et al. 2002). In addition, ParaFit also provides two statistics (ParaFitLink1 and ParaFitLink2), which determine the links that significantly contribute to the cophylogenetic pattern observed (by means of randomization processes of a presence/absence matrix with respect to parasite/host associations).

The Procrustean Approach to Cophylogeny (PACo; Balbuena et al. 2013) is also a global-fit method that assesses similarities between host and parasite trees by comparison of Euclidean embeddings derived from distance matrices. Like ParaFit, it assesses the contribution of each association to the cophylogenetic structure observed. To test for codivergence between two given data sets, PACo uses patristic distances, which are in turn transformed into PCo matrices and are then combined using an association matrix of the parasite/host links. In contrast to ParaFit, PACo assumes that the parasite phylogeny is dependent on the host phylogeny, therefore it scales and rotates the parasite matrix to fit that one of the host. It is thus suitable for systems where the dependence of a phylogeny upon another is assumed (Balbuena et al. 2013), as is true for the chloroplast/host nucleus system. Additionally, it provides a graphical output of the direct contribution of each association to the phylogenetic pattern recovered from the data sets.

One important, but hardly considered, aspect dealing with cophylogenetic distance-based methods (such as PACo and ParaFit) and their efficiency is the type of input (i.e., ultrametric phylograms, additive, and unit branch length trees) employed to perform data analyses. Depending on the kind of tree data set used for analyses, distance-based methods may take into account evolutionary rates when calculating patristic distances between the OTUs. When additive trees are employed for comparison purposes, branch lengths are therefore considered to compute patristic distances, bringing closer OTUs exhibiting short branch lengths and separating those with longer branches (de Vienne et al. 2012). The potential bias resulting from use of contrasting branch lengths has been discussed by some authors (de Vienne et al. 2012; Balbuena et al. 2013), but its influence in the assessment of phylogenetic congruence between data sets has never been tested empirically.

To detect plastid outliers diverging phylogenetically from the evolutionary history of the nuclear host, we here apply the PACo and ParaFit methods to a molecular sequence data set of Catasetinae. This group of orchids encompasses approximately 300 species distributed from Southern Florida to Northern Argentina (Romero and Pridgeon 2009). Previous phylogenetic studies of Catasetinae have only been based on few (if not single) molecular loci and limited taxon samplings (Pridgeon and Chase 1998; Batista et al. 2014; Whitten et al. 2014). Particularly, *Catasetum* Rich ex Kunth, *Cycnoches* Lindl., and *Mormodes* Lindl. are known for the spectacular sexual dimorphism (an exceedingly rare trait among orchids: Pérez-Escobar et al. forthcoming) and a great interspecific variation of floral morphologies. Catasetinae is particularly suitable for our investigation, as natural hybridization because of pollinator sharing

has been reported from *Catasetum* (Dressler 1968a; Romero and Carnevali 1990, 1991, 1992; Romero and Jenny 1992), *Mormodes* (Dressler 1968a), and it might also occur in *Cycnoches* (Pérez-Escobar et al. forthcoming).

Distance-based cophylogenetic analyses have been widely used inside the parasite/host coevolutionary framework, but this is—to the best of our knowledge—the first time that they are applied to test for congruence and detect outlier associations in organelle/host nucleus systems. In this study, we test the effectiveness of PACo and ParaFit by comparing molecular trees separately inferred from organellar and nuclear data sets. Through simulations and analysis of real data sets, we herein show that this approach not only efficiently detects these outlier associations when applied to independently derived organellar and nuclear trees, but also allows the user to evaluate the contribution of each single association in either small or large data sets. We also perform simulations to assess the influence of contrasting branch lengths between trees in distance-based methods such as PACo, using randomly generated additive and congruent unit branch length trees with branches randomly added, which naively recreate taxa that have undergone evolutionary processes such as chloroplast capture after hybridization and ILS occurring at shallow levels of phylogenies.

Workflows for applying cophylogenetic analyses are not standardized at this moment, and we therefore provide a pipeline for managing tree input, executing PACo and ParaFit, and spotting outlier associations from trees or alignments in the public domain software R (R Development Core Team 2015). This pipeline implements a method to identify outlier associations with high reliability based on associate squared residuals produced by PACo exceeding a threshold value. To better orient end-users with little or no experience through the use of the pipeline, a complete tutorial is also provided with a worked example of nuclear ribosomal and chloroplast phylogenies of *Satyrium* Sw., another orchid taxon, in which topological conflict has been reported (van der Niet and Linder 2008).

Material and Methods

Laboratory Techniques, Taxon Sample, and Phylogenetic Analyses

Genomic DNA was extracted from herbarium and fresh leaf material (preserved in silica gel and partly cultivated at the botanical gardens of Hannover and Munich, Germany) with the NucleoSpin®plant kit (Macherey-Nagel; Düren, Germany), following the manufacturer's protocol. We sequenced the nuclear ribosomal external and internal transcribed spacers (ETS and ITS, respectively), the nuclear low copy gene *Xdh*, a ~1500 bp long portion of the chloroplast gene *ycf*1, as well as the *trn*S-*trn*G intergenic spacer. Amplification settings and sequencing primers used for ITS, ETS, *Xdh*, *trn*S–*trn*G, and *ycf*1 are specified in Table S1. PCR products were purified with the ExoSap clean-up kit (Fermentas; St. Leon-Rot, Germany), and sequencing reactions were run on an ABI 3130 capillary sequencer (Applied Biosystems; Carlsbad, CA, USA), following the manufacturer's protocol. Sequence editing was carried out using the trial version of CodonCode Aligner v. 4.0.4. (CodonCode Corporation; Centerville, MA, USA).

We investigated 50 OTUs representing 47 species and covering the known diversity of Catasetinae at the generic and sectional level. In addition, 10 outgroup taxa were included in phylogenetic analyses for rooting purpose. We compiled sequences from six loci, namely ETS+ITS+*Xdh* (consistently treated as "n" in the following) and *mat*K+*trn*S–*trn*G+*ycf*1 ("o"). The concatenated "o" + "n" alignment consisted of 142 empty out of 366 cells. Supplementary Table S2 (available as Supplementary Material on Dryad at http://dx.doi.org/10.5061/dryad.q6s1f) provides an accession list with full species names, geographic origins, vouchered specimens, and GenBank accession numbers (including newly generated sequences) of taxa included in phylogenetic analyses.

We performed two main phylogenetic analyses using data matrices with (i) all "n" OTUs and (ii) all "o" OTUs. Additionally, we performed phylogenetic analyses of each locus for all corresponding OTUs separately. Aligning of single loci was carried out separately using MAFFT version 7.1 (Katoh and Standley 2013; freely available at http://mafft.cbrc.jp/alignment/software/; accessed October 11, 2015) and the default parameters. Data matrices of each locus were concatenated afterwards. For multiple alignments of the nuclear ribosomal loci, the Q-INS-i strategy was employed, which takes secondary structure information into account (Katoh and Toh 2008). Alignments of each locus retrieved from MAFFT were also manually inspected. The complete alignment is available as a *.nex file on Dryad at http://dx.doi.org/10.5061/dryad.q6s1f.

Individual and concatenated analyses were carried out under Bayesian, maximum likelihood (ML), and maximum parsimony (MP) criteria. The best-fitting evolutionary models for Bayesian and ML analyses (for individual data sets) were selected from 56 models implemented in jModelTest version 2.1.3 (Darriba et al. 2012), employing the likelihood ratio test (LRT) and the Akaike information criterion (Supplementary Table S3). Bayesian and ML analyses were implemented in MrBayes version 3.2.2 (Ronquist et al. 2012) and RAxML-HPC Blackbox version 8.0.0 (Stamatakis 2014), respectively, via the CIPRES Science Gateway computing facility (Miller et al. 2010, freely available at http://www.phylo.org). Bayesian inference was carried out performing two independent runs of four Markov chain Monte Carlo (MCMC) analyses with 20 million generations each, sampled every 1000th generation, and using mean default settings and a Dirichlet prior distribution. The performance and convergence of the parameters of the Bayesian inference were checked using the software TRACER version 1.5 (freely available at http://beast.bio.ed.ac.uk/Tracer; accessed October

11, 2015). Statistical support was assessed via 1000 bootstrap replicates. Parsimony ratchet analyses were implemented in Winclada version 1.0 (freely available at http://www.cladistics.com/about_winc.htm; accessed October 11, 2015) using the following settings: heuristic search, uninformative characters deactivated, 500 iterations, holding 1 tree per iteration, ambpoly=default. Statistical support values (BPP: Bayesian posterior probabilities, LBS: ML bootstrap support, PBS: parsimony bootstrap support) were drawn on the resulting, best scoring ML tree.

Testing Divergent Evolution among Chloroplast and Nuclear Data Sets

We assessed the contribution of specific organelle/host nucleus associations to topological conflicts to detect outliers that may correspond to evolutionary events potentially of particular interest using PACo (Balbuena et al. 2013) and ParaFit (Legendre et al. 2002), implemented in the R software packages "ape" v3.0-8 (Paradis et al. 2004) and "vegan" v2.0-9 (Oksanen et al. 2013). To determine whether the chloroplast phylogeny tracks the same phylogenetic history as that of the nucleus, we applied the same principle of parasite/host codivergence to our "o" and "n" data sets (note that this principle is also applicable to "n" and any other organelle genome). Thus, the tree derived from the nuclear sequences is considered the "host" phylogeny, while the tree derived from chloroplast sequences correspond to the "parasite" (or endosymbiont) phylogeny.

To test the null hypothesis that "the similarity between the trees is not higher than expected by chance," we transformed "o" and "n" trees into matrices of patristic distances and applied PACo and ParaFit. Throughout the present study, transformation of patristic distances into the Euclidean PCo space required by both PACo and ParaFit was achieved using the method proposed by de Vienne et al. (2011), which imposes less distortion into the original distances compared with regular eigenvalue corrections. The significance of both tests was established by different permutational approaches (see Legendre et al. 2002; Balbuena et al. 2013 for details) based on 100,000 random permutations of the association matrix. In a first step, we executed PACo and ParaFit using phylograms as input trees. To account for the effect of large distances between particular associations because of highly different branch lengths in the corresponding trees (although the topologies may be identical), we also conducted the tests using unit branch length trees as input. Additionally, PACo and ParaFit analyses were optimized on 10,000 post burn-in trees obtained from Bayesian inferences, to consider the effect of phylogenetic uncertainty and statistical support. Every branch length within these trees was then converted to a value of one (to obtain unit branch length trees) in R using the function *compute.brlen* in package "ape."

In PACo, m^2_{XY} represents the sum of squared residuals of each "o"/"n" association e^2_i. Thus, the latter provides a direct measure of the contribution of each association to the global fit (Balbuena et al. 2013). This measure can be normalized as a proportion of m^2_{XY} (i.e., $\varepsilon^2_i = e^2_i / m^2_{XY}$). In case of perfect congruence between both phylogenies, the ε^2_i's are expected to follow a uniform distribution with expected mean $1/N$, where N = number of "o"/"n" associations. Therefore, $1/N$ provides a threshold value and any ε^2_i linked to a conflicting association is expected to be $> 1/N$. In ParaFit, we used the value of the ParaFitLink2 statistic ($pfl2_i$) to evaluate the contribution of each link association, since it is more appropriate than ParaFitLink1 in one-to-one association scenarios (Legendre et al. 2002). This statistic is constructed similarly to a partial F-statistic and is expected to be ≈ 0 when a given link is conflicting (Legendre et al. 2002).

Both the ε^2_i and $pfl2_i$ statistics were plotted as a vector diagram representing each vector of the "o"/"n" associations, where the magnitude and orientation of each vector would indicate the degree of topological association between the corresponding OTU in the two trees. Ideally, it would produce two distinct groups of vectors representing conflicting and nonconflicting "o"/"n" associations, respectively (see section "Simulations" for details). The efficiency of PACo alone and PACo in combination with ParaFit to separate the two groups of associations was evaluated by the partitioning around medoids (PAM) clustering algorithm (Kaufman and Rousseeuw 1990), as implemented in the R package "cluster" v. 2.0 (Maecheler et al. 2015). In particular, the average silhouette width (Kaufman and Rousseeuw 1990) was used as a measure of the ability to separate congruent and outlier "o"/"n" associations.

Simulations

Gene trees were simulated to determine whether the combined application of PACo and ParaFit or the sole execution of PACo is appropriate to detect divergences between the evolutionary history of "o" and "n." Simulations were carried out as follows:

(1) One thousand random rooted ultrametric trees were generated with the function *evolver* of the software PAML (Yang 2007) using birth and death rates of 0.5 and a sampling fraction of 0.0005. These parameters were deemed realistic and biologically meaningful to generate random phylogenetic trees (Aris-Brosou and Yang 2003). We randomly chose 10 trees of this ultrametric set and simulated sequence evolution with uniform sequence lengths (1000 characters) under the GTR+Γ evolution model, using "phylosim" v. 2.1.1 (Sipos et al. 2011). Two sets of 10,000 post burn-in Bayesian trees were estimated from each simulated alignment,

using the same settings for Bayesian inferences aforementioned.

(2) A subsample of 1000 trees derived from each of the two parallel Bayesian runs was used to represent the shared coevolutionary history of "o" and "n" loci. That is, one tree set was assigned as the organelle phylogeny and the counterpart from the same pair as the "n" phylogeny.

(3) Biological processes rendering phylogenetic distortion in evolutionary history (e.g., hybridization, ILS) were simulated by adding a fixed number of random branches to different nodes in the trees, using the function *add.random* of "phytools" v. 0.4 (Revell 2012). This process was carried out for each "o" and "n" Bayesian set of trees, thereby rendering a pair of additive trees sharing part of their topology but differing in the position and length of the randomly added branches. (We ensured in our simulation that added random branches did not fall in the same positions in both trees.) Thus, each pair can be viewed as a pair of species trees reflecting the evolutionary history of "o" and "n."

(4) Matrices of patristic distances of each additive tree and patristic distance matrices resulting from setting all branch lengths = 1 (i.e., unit branch length trees) were computed.

(5) For each "o"/"n" association, the corresponding ε_i^2 (PACo) and $pfl2_i$ (ParaFit) were computed with both types of distance matrices.

(6) The median ε_i^2's obtained with PACo were centered around $1/N$ and were plotted with the corresponding median $pfl2_i$ values onto a Cartesian plane yielding a vector diagram, where the magnitude and orientation of each vector is expected to be indicative of the degree of topological congruence of each "o"/"n" association.

(7) We performed cluster analysis using the PAM algorithm (Kaufman and Rousseeuw 1990). We aimed at determining the proportion of associations correctly classified as either outliers or congruent OTUs in relation to the phylogeny size and the proportion of outlier/congruent OTUs based on the standardized median values of ε_i^2 and $pfl2_i$. Clustering analyses were carried out using two clusters (k=2) (occasionally, three clusters were used because in some instances *pfl2* tended to split congruent associations in two unnatural clusters, Supplementary Table S5). The efficiency of the classification procedure was evaluated by means of the proportion of congruent, incongruent, and overall associations correctly classified as well as by the average silhouette width value (Kaufman and Rousseeuw 1990).

The following parameter combinations were used for all simulation approaches:

a) Trees with 50 OTUs and 10%, 20%, 30%, and 40% of random branches.

b) Trees with 100 OTUs and 10%, 20%, 30%, and 40% of random branches.

c) Trees with 200 OTUs and 10%, 20%, 30%, and 40% of random branches.

Using PACo Pipeline to Test for Topological Congruence and Detecting Outlier Associations

We provide a pipeline to assess cophylogeny, in terms of topological congruence, between "o" and "n" phylogenies, and readily identify outlier taxa in both phylogenies. The pipeline is based on PACo, ParaFit, and other set of R functions applied in the packages "ape," "cluster," "gplots" v. 2.17 (Warnes et al. 2011), "phytools," and "vegan." It allows the user to convert phylograms to trees with unit branch lengths (when necessary, see section "Discussion") and to display outlier associations detected by PACo independently on trees derived from each data set analyzed. It only requires a recent version of R and the aforementioned packages installed on the machine. The pipeline is also available on Dryad at http://dx.doi.org/10.5061/dryad.q6s1f.

RESULTS

Testing PACo and ParaFit with Simulated Data: Identification of Outlier Associations

A total of 240 simulations were executed, yielding comparable results. The efficiency of classification decreased overall with phylogeny size and proportion of outlier taxa, and the best results were obtained with additive trees (Fig. 1). Our approach to detect conflicting associations using PACo combined with ParaFit statistics showed high efficiency, particularly in simulations with additive trees, where the number of outliers was $\leqslant$20% of the total number of OTUs. In these simulations, involving phylogenies of 200, 100, and 50 OTUs, PAM clustering correctly identified 1040 of 1050 outliers and all 5950 nonconflicting associations based on the median values of the ε_i^2 and $pfl2_i$ statistics (Fig. 1, Supplementary Figs S1 and S2, Supplementary Table S4). Nonetheless, applying solely PACo for outlier detection with PAM to the same phylogenies increased the number of correctly identified outlier associations to 1048.

In general, using PACo alone yielded better classification results. Under simulations of phylograms with 100 OTUs (30 outliers), PACo misidentified, for instance, 4 outliers versus 12 using PACo+ParaFit (Fig. 2). In addition, the average silhouette width values of classifications involving PACo were higher, indicating a stronger clustering structure in all simulations, than

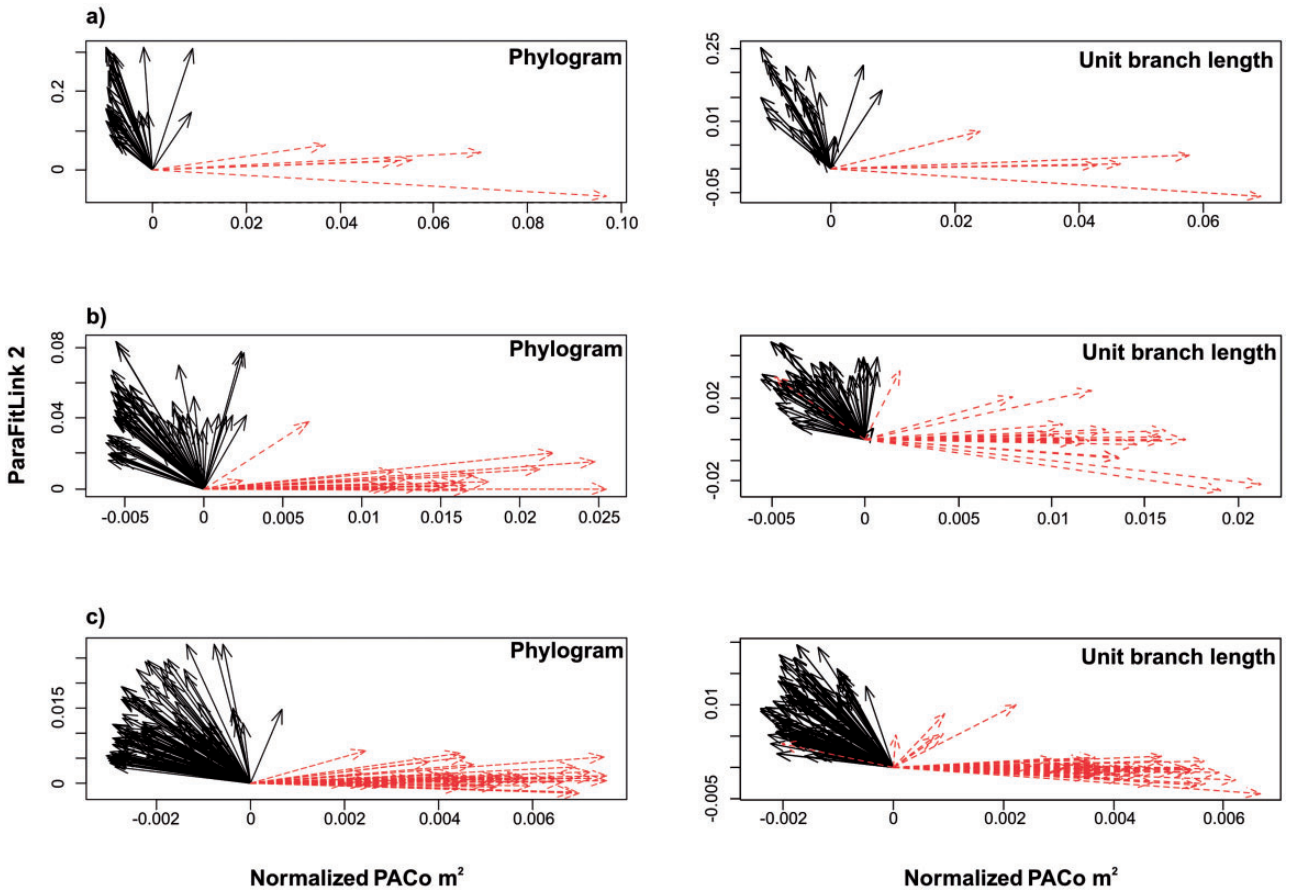

FIGURE 1. Vector diagrams of squared residual values ε_i^2 and ParaFitLink2 statistic (*pfl2*) obtained by PACo and ParaFit using simulated data, respectively. Vector magnitude and orientation is relative to the topological degree congruence of each "o"/"n" association. Outlier sequences are shown in red, dashed lines, whereas nonconflicting associations are shown in black. a) Additive trees (left) and unit branch length trees (right) with 50 terminals (5 outliers); b) with 100 terminals (20 outliers); c) with 200 terminals (60 outliers).

using PACo+ParaFit (Supplementary Table S4). In particular, the average silhouette values obtained with PACo alone ranged from 0.80 to 0.94 in simulations, where the number of outliers was $\leqslant$20% of the total number of associations, whereas the corresponding range using PACo+ParaFit was 0.50–0.80.

Phylogenetic Incongruence within Catasetinae

In this study, 180 new sequences were generated (Supplementary Table S2). The concatenated "n" alignment was 2171 bp in length and included 584 parsimony informative positions, while the concatenated "o" data set was 4300 bp long comprising 392 parsimony-informative positions (Supplementary Table S5). Bayesian, ML, and MP trees of individual data partitions recovered similar topologies (not shown). These reconstruction methods provided maximal support for the monophyly of Catasetinae as well as the subordinate (generic) lineages *Cyanaeorchis* Barb.Rodr, *Grobya* Lindl., *Galeandra* Lindl., and core Catasetinae (i.e., *Catasetum*, *Clowesia* Lindl., *Cycnoches*, *Dressleria* Dodson, and *Mormodes*) (Fig. 3). Additionally, they recovered very similar topologies at the backbone placing almost all generic lineages (except *Galeandra*) in equal phylogenetic positions.

Several conflicting and highly supported phylogenetic placements were present within *Catasetum*, *Cycnoches*, and *Mormodes* (conflicting associations highlighted red and in black boldface in Fig. 3). Particularly, *Cycnoches* was subjected to significantly diverging topologies while comparing separately derived trees: Both data sets retrieved two primary and maximally supported subclades, whose compositions were different for a number of OTUs. The most prominent example is *Cycnoches haagii* Barb. Rodr.: It was sister species of the remainder of *Cycnoches* in the "n" tree (1.00 BPP, 100 LBS, 100 PBS), whereas it appeared embedded within one of the two strongly supported subclades of *Cycnoches* (1.00 BPP, 99 LBS, 66 PBS) in the "o" phylogeny. Another striking example of a taxon reconstructed as conflicting with high statistical support was *Cycnoches lehmannii* Rchb.f.: It was placed as sister species to one of the two clades present in *Cycnoches* in the "n" phylogeny (1.00 BPP, 100 LBS, 100 PBS) whereas in the "o" tree, it clustered together with *Cycnoches ventricosum* Bateman in a strongly supported clade (0.99 BPP, 82 LBS, 82 PBS).

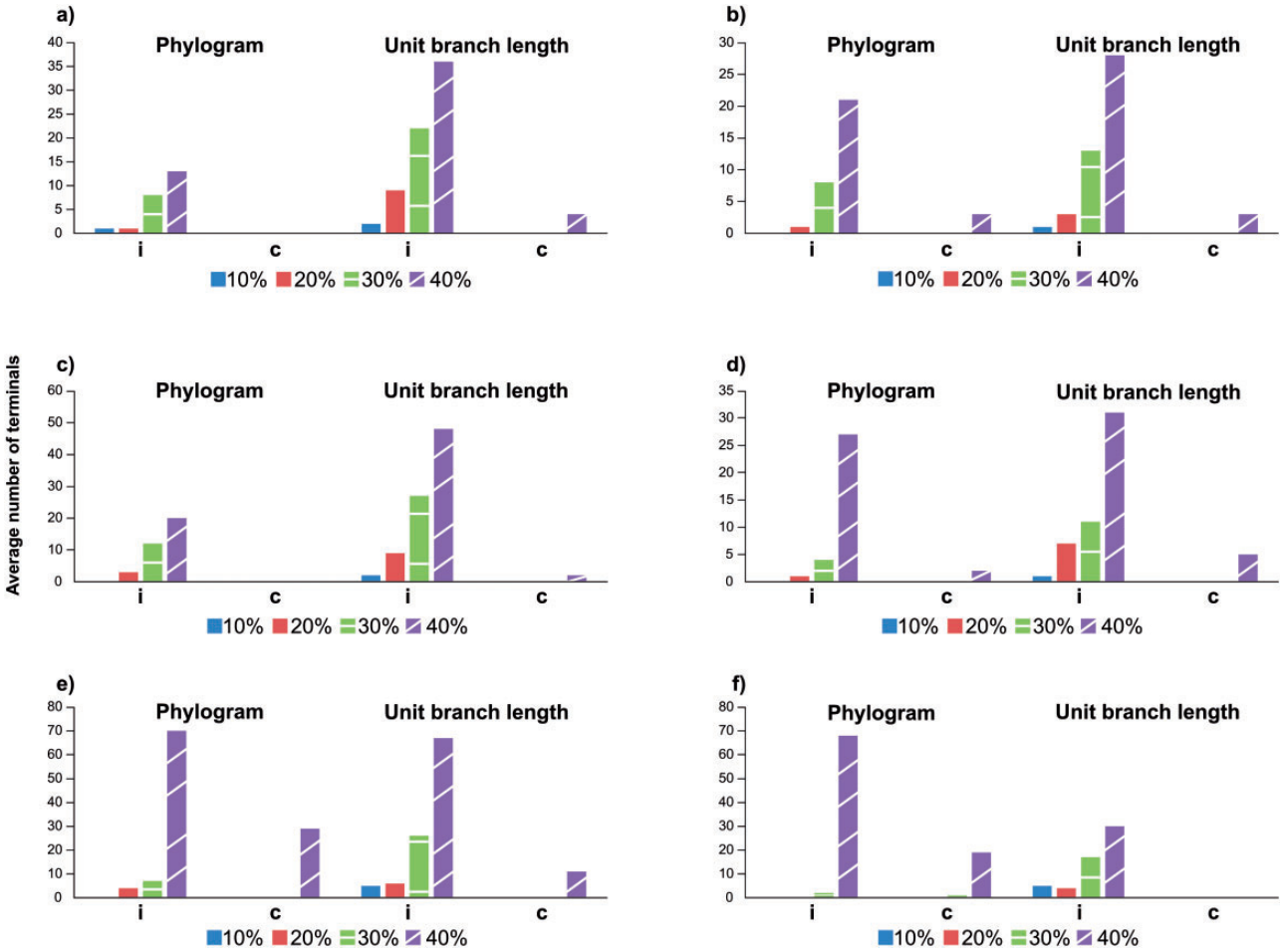

FIGURE 2. Average number of outlier (i) and congruent terminals (c) misidentified by PACo (right) and PACo+ParaFit (left) approaches using a)–b) Additive trees and cladograms with 50 terminals; c)–d) 100 terminals; e)–f) 200 terminals. Proportion of outlier OTUs included in trees are color-coded: blue, 10% of total number of tree terminals; red, 20%; green, 30%; purple, 40%.

Testing PACo and ParaFit Using Real Data: Detection of Outliers Between Trees Independently Derived from Plastid and Nuclear Loci

Using both ML phylograms and unit branch length trees, statistical significance of the global value of PACo and ParaFit yielded comparable results rejecting H_0 ($P = 0.0001$ and $P = 0.001$, respectively) and thus indicating that the "o" and "n" phylogenies were to some extent reflecting phylogenetic congruence. When using ML phylograms of the "o" and "n" data sets as inputs, PACo showed 21 associations, whose median squared residuals ε_i^2 were higher than the cutoff value $1/N$. Seventeen (out of 26 true) recognized outliers were in fact linking conflicting taxa (sequence names highlighted in red in Fig. 3). Therefore, these associations were confidently identified as potential outlier taxa (Fig. 4a). In contrast, when PACo was applied to the "o" and "n" unit branch length trees using the same threshold value, 25 associations (of which 20 clearly presented conflicting positions in both trees) were identified as incongruent (indicated in red in Fig. 4b). Thus, PACo erroneously identified slightly more potential outliers when using unit branch length trees than additive trees. Surprisingly, some conflicting associations that were not successfully identified by PACo using additive trees were indeed recovered as such when analyzing unit branch length trees (e.g., "o"/"n" associations of *Catasetum* x *roseoalbum* (Hook.) Lindl., *Cycnoches guttulatum* Schltr., Fig. 4b). In addition, associations identified by PACo with the highest residual squared scores were those that showed the most incongruent positions between "o" and "n" trees. For instance, the three outlier OTUs *C. haagii*, *Galeandra devoniana* M.R.Schomb. ex Lindl., and *Galeandra* sp. 92 having conflicting, well-supported phylogenetic positions in "o" and "n" trees also showed the highest contributions to the normalized squared residuals (Fig. 4a, see above).

Detection of outliers in ParaFit was not as efficient as in PACo, using either additive or unit branch length trees as input data. Most of the links retrieved by ParaFit as putative outliers were actually OTUs that were not

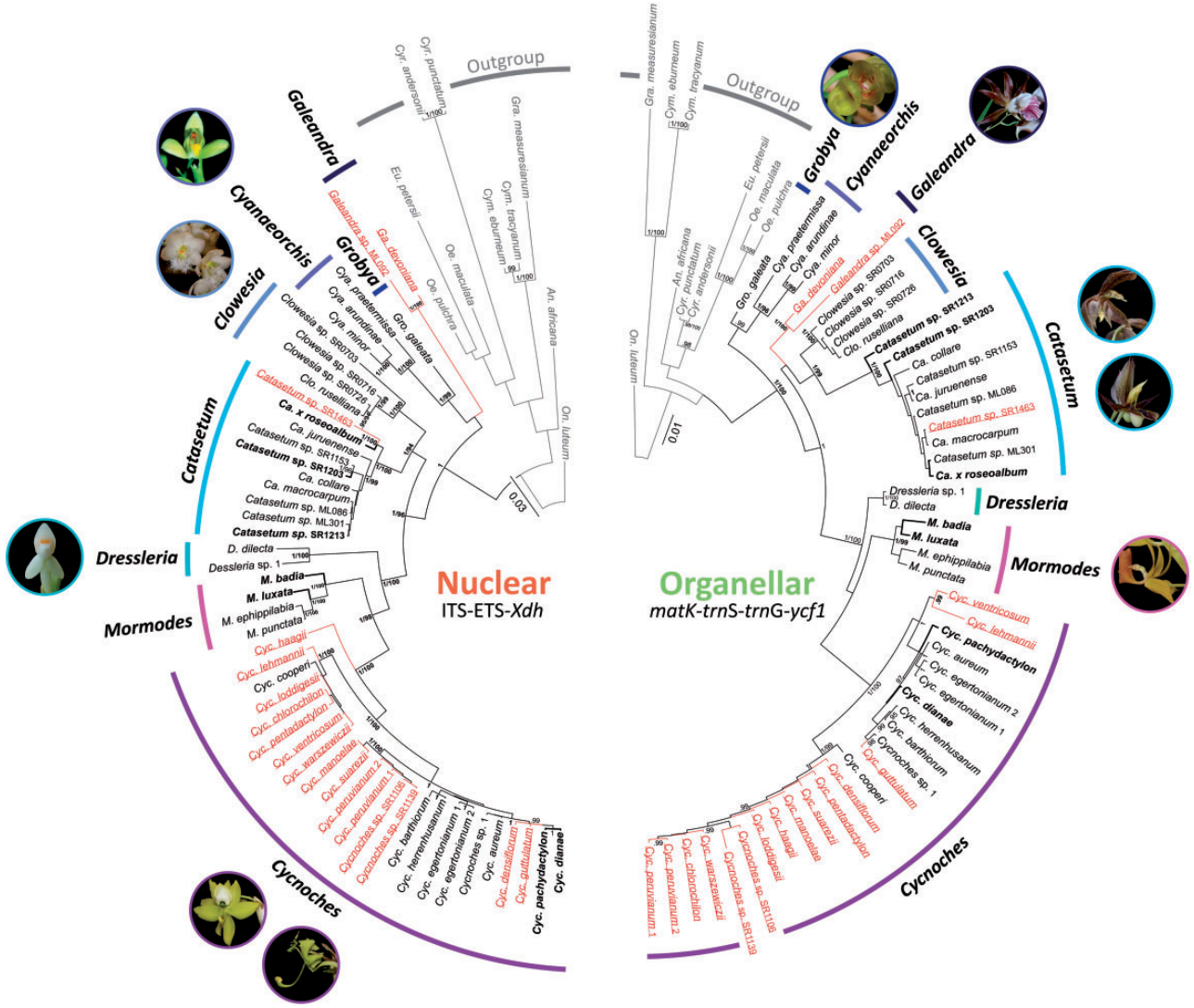

FIGURE 3. Phylogenetic relationships of Catasetinae showing outlier taxa in *Catasetum*, *Cycnoches*, and *Mormodes* between ML trees independently derived from "o" (*matK*, *trn*S-*trn*G, *ycf*1) and "n" (ETS, ITS, *Xdh*) data sets. Outgroup taxa are highlighted in gray. Outlier taxa successfully identified by PACo using phylograms as input are highlighted in red and underlined. Conflicting taxa not retrieved by PACo are indicated in bold letters. Numbers on nodes indicate Bayesian posterior probabilities (BPP > 0.90) and ML bootstrap values (LBS > 90). Support values in bold indicate parsimony bootstrap support values (PBS > 70). Photos of *Galeandra* and *Cyanaeorchis* taken by G. Gerlach (Munich) and E. Pansarin (São Paulo).

reconstructed with conflicting positions. In addition, only a small proportion of truly conflicting associations were recovered as outliers (Fig. 5). When using additive trees as input data, for instance, seven of the associations recovered by ParaFit as possible outliers (i.e., with the highest ParaFitLink2 values, Fig. 5a) were actually not conflicting associations. In addition, one of the most divergent terminals (*C. haagii*) between the "o" and "n" phylogenies was indicated as putatively not conflicting (i.e., very low $pfl2_i$ value). Similar results were obtained when ParaFit was executed using unit branch length trees (Fig. 5b). For example, the species *Catasetum collare* Cogn. (nonconflicting between "o" and "n") yielded one of the highest $pfl2_i$ values, thus wrongly indicating an outlier.

The OTU classification executed by PACo and PACo+ParaFit methods using additive trees was validated by the PAM approach. Under the PACo+ParaFit method, 38 OTUs were classified as outliers (Fig. 6a). In addition, congruent and outlier associations were classified into two weak cluster structures (silhouette width value = 0.40). In contrast, classifications carried out solely with PACo (Fig. 6b) yielded comparable results to those obtained by PACo ε_i^2 statistics using $1/N$ as cutoff value (see Fig. 3). Under this method, 19 OTUs were positively classified as outliers, and congruent and outlier associations were separated into two reasonable cluster structures (silhouette width value = 0.63, to be correct as $S = 0.625$) (Fig. 6b).

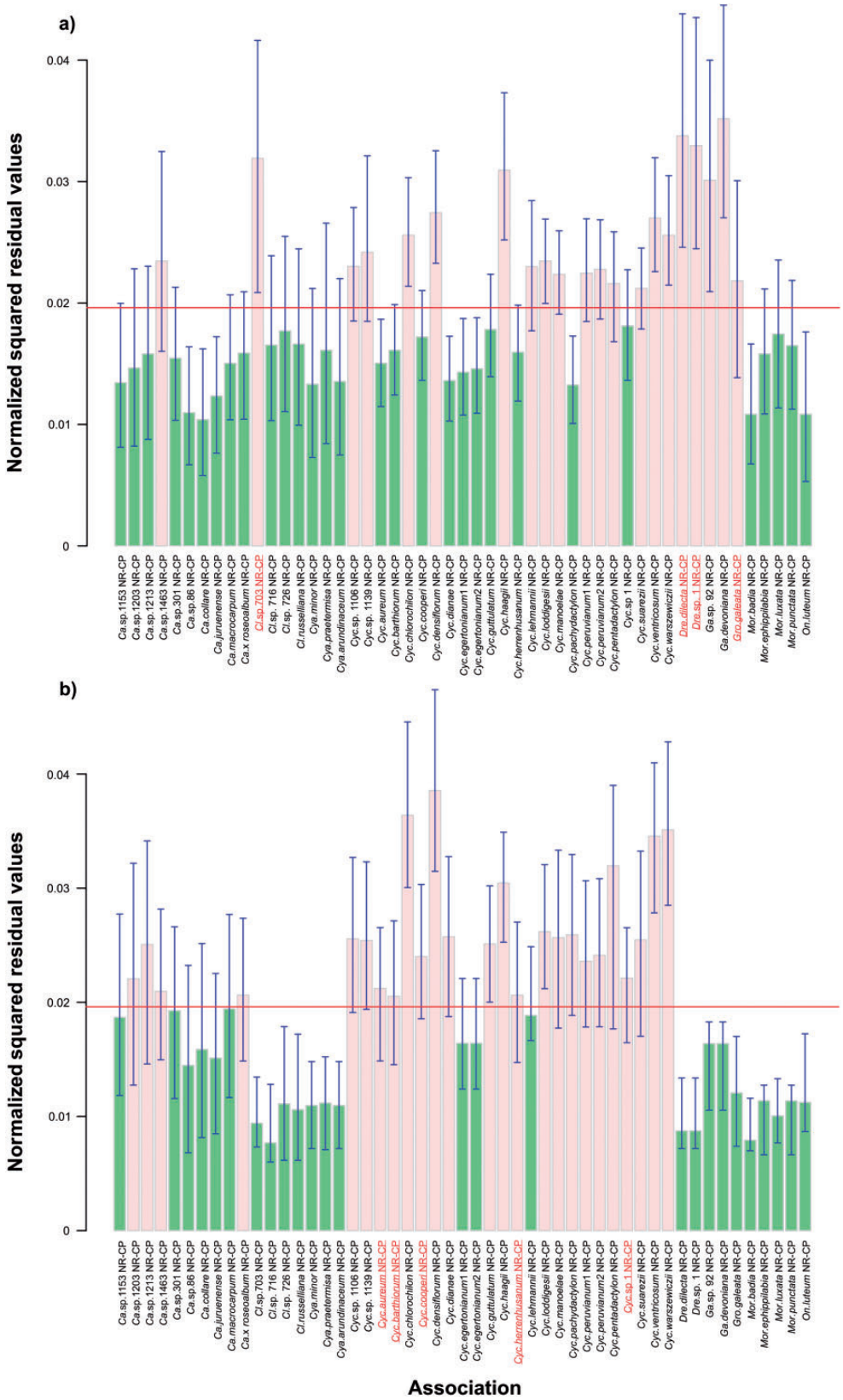

FIGURE 4. Normalized squared residual values ε_i^2 of individual "o"/"n" associations obtained by PACo using a) phylograms and b) unit branch length trees. Associations with squared residual values above the threshold (pink bars) are links identified by PACo as outliers. Outlier associations identified by PACo that do not have conflicting positions in phylogenies are shown in underlined red letters.

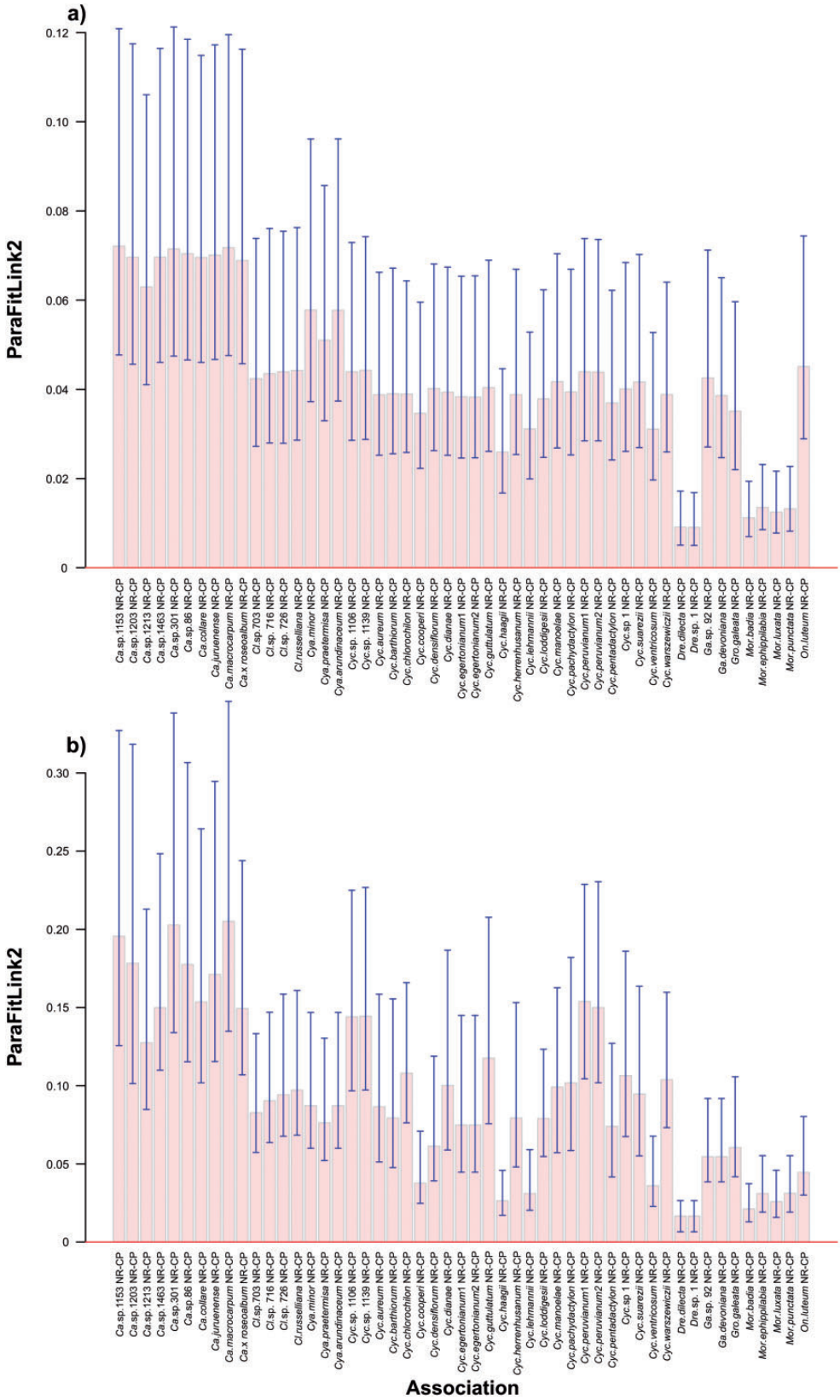

FIGURE 5. ParaFitLink2 ($pfl2_i$) statistic of individual "o"/"n" associations obtained by ParaFit using a) phylograms and b) unit branch length trees.

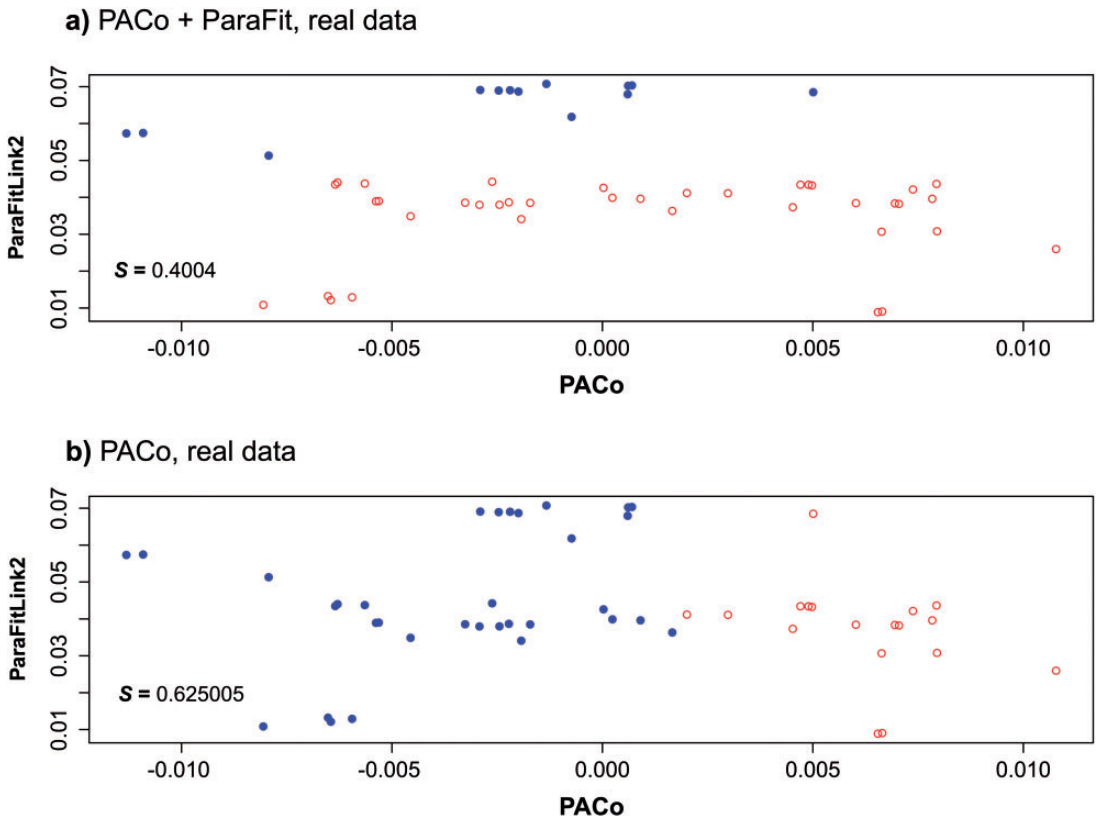

FIGURE 6. Cluster plots of outlier (red, hollow circles) and congruent (blue, filled circles) terminals classified by PACo+ParaFit (a) and PACo (b) methods validated using PAM algorithm. Silhouette width values (S) of each cluster analysis are also provided (inset).

DISCUSSION

Cophylogeny between Chloroplast and Nuclear Loci with Detection of Outlier Associations

Chloroplasts (like other cellular organelles such as mitochondria) are to be interpreted as endosymbionts, having their own (reduced) genome and ribosome type. Their evolution is strongly linked to that of the host cell, but differing substitution rates of nuclear and chloroplast loci (Wolfe et al. 1987; Tepe et al. 2011), their structurally independent replication (Possingham 1980; Heinhorst and Cannon 1993), and other biological processes (e.g., HGT during hybridization: Rieseberg and Soltis 1991) may lead to divergent evolution and incongruence between topologies inferred from organellar and nuclear loci. This is statistically demonstrated by the results of our study, in which we present highly supported but contradicting topologies for a number of associations while comparing nuclear with chloroplast molecular trees. To the best of our knowledge, the extreme degree of incongruence within *Cycnoches* has not been shown for any other plant lineage before; divergent nuclear and plastid topologies are usually moderately if at all statistically supported (Carlsward et al. 2006; Fehrer et al. 2007; Koehler et al. 2008; van der Niet and Linder 2008).

To investigate the phenomenon in detail, we here have tested some cophylogenetic tools traditionally applied to parasite/host systems. Based on the same principle of coevolution (defined as the extent, to which the host and parasite phylogenies are congruent, as inferred by methods such as PACo and ParaFit), we have determined the degree of topological congruence between phylogenies independently derived from "o" and "n" data sets. More importantly, we are not only in search of the single ("true" species) tree (that can be probably more effectively done with software programs such as BUCKy: Larget et al. 2010 and MP-Est: Liu et al. 2010), but we aim at inferring and explaining two (gene) trees that are mostly congruent, but in particular cases not. We thus seek to detect and assess the contribution of each outlier association to the phylogenetic relationships observed that may correspond to exceptional evolutionary events (such as chloroplast capture as result of HGT) in case of conflicts.

It must be acknowledged that in our simulation approaches, we have naively reproduced evolutionary events responsible for topological incongruence (i.e., hybridization, HGT, ILS) by randomly adding a certain amount of OTUs to congruent trees. This of course does not perfectly simulate, for instance, ancient ILS processes, which are known to have occurred in several seed plant lineages (e.g., *Ceanothus*: Hardig et al. 2000; *Juniperus*: Terry et al. 2000; *Hieracium*: Fehrer et al. 2009), especially those that have diversified following rapid radiations (Degnan and Rosenberg 2009). Therefore,

further efforts should be directed toward a more thorough simulation of evolutionary events responsible for topological incongruence that have taken place at deeper phylogenetic levels, and their impact on the performance of the approach here described.

While comparing "o" and "n" DNA trees, all major lineages of Catasetinae are monophyletic and their phylogenetic relationships (except *Galeandra*) are not conflicting, reflecting overall cophylogeny of the corresponding loci. This is also statistically supported by the rejection of H_0: topological incongruence by PACo and ParaFit. The outliers identified by PACo affect *Catasetum*, *Cycnoches*, and *Mormodes*, in which major incongruences between the "o" and "n"data sets have been detected. Natural hybridization has been reported from *Catasetum* (Dressler 1968b; Romero and Carnevali 1990, 1991, 1992; Romero and Jenny 1992), and it might occur in *Cycnoches* as well (Pérez-Escobar et al. forthcoming). Hybridization may lead to the introgression of a chloroplast genome (and hence to a HGT process) from one lineage into another represented by the phylogeny of the nucleus (i.e., chloroplast capture: Tsitrone et al. 2003). Chloroplast capture is often proposed as the explanation for topological incongruence between chloroplast and nuclear phylogenies (Rieseberg and Soltis 1991; Stegemann et al. 2012), and it has also explanatory power for our observations.

In some of our simulations, a number of outlier associations have not distinguished from the nonconflicting counterparts based on the values of their normalized squared residuals (PACo) and ParaFitLink2 statistics. However, our method is highly efficient when applied to large phylogenies with a moderate through low number of outliers (over 99% of incongruent links identified). This range of conditions may reflect in fact characteristics observed in real data sets (Fehrer et al. 2007; Koehler et al. 2008; van der Niet and Linder 2008) that end-users encounter when analyzing cophylogeny between organellar and nuclear data sets. Nevertheless, outlier detection using the Catasetinae data set was not so efficient, when the number of putative outlier associations accounts for up to 40% of the total number of 51 OTUs. The efficiency with these data is comparable to that observed in our simulations with 30% and 40% of added outliers (Fig. 2, Supplementary Table S4).

Classification of OTUs via clustering analysis stands as a useful, complementary tool to validate, how PACo performs retrieval of outliers. As demonstrated by our simulated and real data sets, terminal classifications are more reliable than those executed by PACo combined with ParaFit. Therefore, our method based on representation of phylogenetic relationships in Euclidean space is appropriate to capture properties of tree topologies, even though tree space (except for ultrametric tree space, see Pavoine et al. 2005) is not Euclidean (Cavalli-Sforza and Edwards 1967; Kidd and Sgaramella-Zonta 1971; Holmes 2005). Nevertheless, validation using PACo in combination with ParaFit might be considered for implementation, as it allows the end-user to easily visualize on a Cartesian plane the relationships between the "o"/"n" associations and determine "by eye" (under some data circumstances) groups of outlier and congruent associations (as in Fig. 1).

Effect of Input Data in Distance-Based Methods

PACo and ParaFit may be susceptible to differences in evolutionary parameters of sequences, if patristic distances derived from additive trees are used as input. Although the use of patristic distances obtained from additive trees affords incorporation of evolutionary rates, it may also introduce artifacts in cophylogenetic analyses, such as the attraction of OTUs with short branch lengths and the departure of those exhibiting longer branches. In contrast, when distances derived from pure topologies (e.g., unit branch length trees) are employed, rates of evolution are not considered (de Vienne et al. 2012). To the best of our knowledge, the effect of input data (and hence branch lengths) in cophylogenetic distance-based methods has not been investigated, using extensive simulation approaches and real data, until the present study. Nevertheless, it is still unclear how input data might affect cophylogenetic distance-based methods under different conditions, such as contrasting substitution rates between parasite/host phylogenies (as observed in parasitic plants, which exhibit accelerated substitution rates: Bromham et al. 2013; Bellot and Renner 2014). Further research should focus on simulation approaches, in which contrasting substitution rates between data sets are reproduced in detail, allowing assessment of distance-based methods' performance under these circumstances.

The effect of input data is statistically demonstrated by simulations using patristic distances obtained from additive trees, in which PACo is more efficient overall retrieving outliers from nonconflicting associations than in simulations based on unit branch length trees. Nevertheless, an interesting pattern was observed when PACo was applied to real data, in which lineages with strongly differing branch length such as *Dressleria* and *Grobya* are recognized by PACo as outliers when using additive trees, even in the absence of phylogenetic conflict. Using unit branch length trees as input data can also be a reasonable alternative to evaluate the potential impact of contrasting branch lengths in the phylogenetic congruence context. In addition, it might be useful to detect putative conflicting associations that are not retrieved as such when analyzing phylograms (as observed in our analysis of real data). Our simulations indicate a reduced ability to detect conflicting links, but the efficiency is still acceptable if phylogenies are large and the number of such links is relatively low.

Handling PACo and ParaFit for the Pipeline

Distance-based methods such as PACo and ParaFit are traditionally used in cophylogenetic studies, but

such approaches have not been employed before to comparative tree topologies in organelle/host nucleus systems. To execute both methods, a set of three input files are required: one set of organellar and one set of nuclear trees and a binary association matrix (see Balbuena et al. 2013), in which all sequence names from both trees are included and linked. With prior knowledge on the evolutionary rates of the data set, the user may decide to use unit branch length or directly additive trees as input data. End-users might well run analyses using both kinds of input data for comparative purposes, although our results with real data indicate that the latter option is likely to produce more reliable results.

Workflows for applying cophylogenetic analyses are not standardized yet and therefore, we provide a pipeline for managing input data (i.e., transforming additive trees to unit branch lengths when desired), applying PACo function and spot outliers on error bar plots as well as directly on phylogenies in the software R (available as an R script on Dryad at http://dx.doi.org/10.5061/dryad.q6s1f; accessed October 11, 2015). Owing to the fact that weakly supported and internally unresolved clades may produce artifacts in distance-based methods, the present method accommodates phylogenetic uncertainty by including in the analysis sets of trees derived either from ML or Bayesian phylogenetic inferences. In addition, it readily generates the binary matrix required to execute PACo and eventually identifies outlier associations based on $1/N$ as threshold value as outlined above. A complete tutorial is provided at http://www.uv.es/cophylpaco/; accessed October 11, 2015.

Supplementary Material

Data available from the Dryad Digital Repository: http://dx.doi.org/10.5061/dryad.q6s1f.

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Rumbling orchids: How to assess divergent evolution between the nuclear host and chloroplast endosymbionts

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Supporting information

including: User guide – managing the pipeline in R. A tutorial to execute the pipeline described in the main text is provided, using as a working example conflicting chloroplast and nuclear phylogenies of *Satyrium* (Orchidaceae).

including: additional Figures S1–S6.

including: additional Tables S1–S5.

USER GUIDE – MANAGING THE PIPELINE IN R

An R (R Development core team 2015) script is presented to carry out tests of phylogenetic congruence, and detection of outlier associations, between trees derived from organellar and nuclear loci. The script stands as a pipeline to execute PACo (Procustes Approach to Phylogeny: Balbuena et al. 2013) and ParaFit (Legendre et al. 2002) that are traditionally employed in coevolutionary studies. In addition, it also includes a set of functions useful to spot outliers in trees.

To be executed, the pipeline requires two sets of posterior probability trees derived from Bayesian inference or Maximum Likelihood (ML) phylogenies, corresponding to the organellar and nuclear trees, respectively. The user can decide to run PACo and ParaFit either with phylograms or unit branch length trees as input, in order to take into account and compare the effects of considering evolutionary rates. PACo yields a residual sum of squares (m_{XY}^2) that measures congruence between phylogenies and uses a permutation approach to test significance. Squared residual values (e_i^2) provide a direct measure of each 'o'-/'n'-association's contribution to the global value m_{XY}^2. This estimate can be normalized as a proportion of m_{XY}^2 (i.e., $\varepsilon_i^2 = e_i^2 / m_{XY}^2$). In case of complete congruence between both phylogenies, the ε_i^2's are expected to follow a uniform distribution with expected mean 1/N, where N = number of 'o'-/'n'-associations. Therefore, 1/N provides a threshold value and any ε_i^2 linked to a conflicting association is expected to be > 1/N.

As for ParaFit, the pipeline computes the ParaFitLink2 statistic ($pfl2_i$), which also evaluates the contribution of each link association and is more appropriate than ParaFitLink1 in one-to-one association scenarios (Legendre et al. 2002). The $pfl2_i$ value of a given association is inversely proportional to the phylogenetic pattern observed. Therefore, outlier sequences are expected to have $pfl2_i \approx 0$. The pipeline produces plots of the median and 95% empirical confidence intervals of ε_i^2 and $pfl2_i$ values, and outlier associations can be identified by comparison with a given cut-off value. Because in all simulations and real data set analyses PACo performed better than the *pfl2* statistic, the respective ε_i^2 value of each association only is plotted independently onto the nuclear and organelle phylogenies, thus providing a visual detection of outliers for the end-user.

In order to assist users with little or no experience about R, we provide herein a tutorial to the pipeline. All analyses can be executed by cutting and pasting the syntax in an R console. The text in red represents parameters that should be set by the user in order to

adapt the analysis to specific purposes. The tutorial demonstrates the efficiency of PACo and the pipeline to detect outlier associations and to test for congruence using the plastid (*mat*K, *trn*L–*trn*F, *trn*S–*trn*G) and nuclear-ribosomal (ITS) phylogenies of *Satyrium* Sw. (Orchidaceae), for which topological conflicts between trees derived from nuclear and plastid data sets have been reported (van der Niet and Linder 2008). We have made available separate chloroplast and nuclear derived posterior probability trees (Dryad repository, doi:10.5061/dryad.q6s1f) used throughout this tutorial, and a chloroplast-/nuclear concatenated alignment is available at TreeBASE (Study ID S1221).

RUNNING PROCEDURE

In addition to the basic R installation, five dedicated packages need to be installed to implement the pipeline, namely "ape", "cluster", "gplots", "phytools", and "vegan" (see http://cran.r-project.org/doc/manuals/R-admin.html#Installingpackages for details). For every running analysis, libraries required to execute the pipeline must be loaded.

```
library (ape)
library (cluster)
library (gplots)
library (phytools)
library (vegan)
```

PACo application

A complete description of PACo is provided by Balbuena et al (2013), and we refer to this study for details describing syntaxes of functions. To execute PACo and ParaFit, a set of functions have to be defined first. In both cases, the method proposed by de Vienne et al. (2011) is used to transform of patristic distances into Euclidean space.

```
PACo.dV <- function (H.dist, P.dist, HP.bin) {
  HP.bin <- which(HP.bin > 0, arr.in=TRUE)
  H.PCo <- pcoa(sqrt(H.dist), correction="none")$vectors
  P.PCo <- pcoa(sqrt(P.dist), correction="none")$vectors
  H.PCo <- H.PCo[HP.bin[,1],]
  P.PCo <- P.PCo[HP.bin[,2],]
  list (H.PCo = H.PCo, P.PCo = P.PCo)
}
```

The function *D.wrapper* will execute PACo and ParaFit for each of the trees included in the tree data sets (see below). It also allows the end-user to compare the influence of evolutionary distances in Procrustes and ParaFit analyses by executing PACo using either phylograms or unit branch length trees as input data. Unit branch length trees are obtained by computing branch lengths values of 1 to each branch of the tree data sets.

```
D.wrapper <- function(n) {
  DH.add <- cophenetic(treeH[[n]])
  DP.add <- cophenetic(treeP[[n]])
  DH.top <- cophenetic(compute.brlen(treeH[[n]], 1))
  DP.top <- cophenetic(compute.brlen(treeP[[n]], 1))
  DH.add <- DH.add[rownames(NCP),rownames(NCP)]
  DP.add <- DP.add[colnames(NCP), colnames(NCP)]
  DH.top <- DH.top[rownames(NCP),rownames(NCP)]
  DP.top <- DP.top[colnames(NCP), colnames(NCP)]

  PACo.add <- PACo.dV(DH.add, DP.add, HP)
  Proc.add <- procrustes(PACo.add$H.PCo, PACo.add$P.PCo)
  add.res <- residuals(Proc.add)
  HostX <- Proc.add$X
  ParY <- Proc.add$Yrot
  colnamesPACo <- paste(rownames(HostX),rownames(ParY), sep="_")

  PACo.top <- PACo.dV(DH.top, DP.top, HP)
  Proc.top <- procrustes(PACo.top$H.PCo, PACo.top$P.PCo)
  top.res <- residuals(Proc.top)

  PF.add <- parafit(sqrt(DH.add), sqrt(DP.add), HP, nperm=1,
test.links=TRUE, silent=TRUE)
  PFL2.add <- c(PF.add$link.table[,5])

  PF.top <- parafit(sqrt(DH.top), sqrt(DP.top), HP, nperm=1,
test.links=TRUE, silent=TRUE)
  PFL2.top <- c(PF.top$link.table[,5])

  write (add.res, file="PACo_res_add.txt", ncolumns = NLinks ,
append=TRUE, sep="\t")
  write (top.res, file="PACo_res_top.txt", ncolumns = NLinks ,
append=TRUE, sep="\t")
  write (PFL2.add, file="PFL2_add.txt", ncolumns = NLinks ,
append=TRUE, sep="\t")
```

```
  write (PFL2.top, file="PFL2_top.txt", ncolumns = NLinks ,
append=TRUE, sep="\t")
  write (colnamesPACo, "colnamesPACo.txt", ncolumns=NLinks,
sep="\t")
}
```

Data input

In order to execute the global test of congruence, two files must be loaded, namely consensus trees derived from the organellar and nuclear data sets. For example, the consensus trees produced by the MrBayes application are to be used in this step. In addition, a set of posterior probability trees obtained from Bayesian analysis or ML trees derived independently from the organellar and nuclear data sets are required for detection of outlier associations. Using a tree set and not consensus tree for outlier detection is preferred, because the former option allows for inclusion of phylogenetic uncertainty into the analysis. Trees may be uploaded in either Nexus or Newick format. A third file required to execute PACo and ParaFit is a binary matrix, in which corresponding pairs of organellar and nuclear Operational Taxonomic units (OTUs) are associated. However, this matrix is readily generated by the pipeline (see below) when both data sets share exactly the same number and names of OTUs. The user should ensure that sequence names in the binary association matrix match exactly with those of the trees. (Note also that the order of the taxa in the phylogenies should match with that of the binary matrix, but the pipeline includes a sorting algorithm to ensure this and no user intervention is required in this regard.) If data sets contain unequal numbers of sequences, then end-users must generate and upload the association matrix manually. Note that input files should include OTU labels that match exactly in all files, and we recommend the use of short name labels for the sake of the interpretation of graphical outputs. Use the following syntax to load trees in R:

```
NTree <- read.tree("myfilename.t")
CPTree <- read.tree("myfilename.t")
```

If input phylogenies are instead in Nexus format:

```
NTree <- read.nexus("myfilename.t")
CPTree <- read.nexus("myfilename.t")
```

For large data sets (e.g., trees with more than 200 OTUs), manual generation of the binary association matrix comprising organellar and nuclear OTUs can be time-consuming. The binary matrix can be generated by the following code:

```
NTaxa <- sort(NTree$tip.label)
CPTaxa <- sort(CPTree$tip.label)
NCP <- as.matrix(table(NTaxa, CPTaxa))
```

However, if small trees (e.g., trees with less than 50 OTUs) are being analyzed, or if the user already has a text file with the association matrix, it can be loaded into R:

```
NCP <- as.matrix(read.table("myfilename.txt", header=TRUE))
```

In order to accommodate for phylogenetic uncertainty into the analysis, a sets of trees in either Nexus or Newick format is required for detection of outlier sequences (see above):

```
ByH <- "myfilename.t"
ByP <- "myfilename.t"
```

Trees in Newick format

```
treeH <- read.tree(file= ByH)
treeP <- read.tree(file= ByP)
```

Trees in Nexus format

```
treeH <- read.nexus(file= ByH)
treeP <- read.nexus(file= ByP)
```

Using the following script, the end-user may set a given number of trees to be discarded (burn-in) from the tree data set, in this example the first 18,000 trees are discarded:

```
treeH <- treeH[18001: length(treeH)]
treeP <- treeP[18001: length(treeP)]

NLinks = sum(NCP)
HP <- diag(NLinks)
```

Testing cophylogeny between nuclear and chloroplast phylogenies

To execute the global test of congruence between organellar and nuclear data sets, PACo requires patristic distances to obtain a global m^2_{XY} value. Therefore, consensus organellar and nuclear trees (see data input) must be transformed into matrices of patristic distances:

```
N.D <- cophenetic (NTree)
CP.D <- cophenetic (CPTree)
```

The organellar and nuclear matrices of patristic distances are then sorted to match the rows and the columns of the binary association matrix:

```
N.D <- N.D[rownames(NCP),rownames(NCP)]
CP.D <- CP.D [colnames(NCP), colnames(NCP)]
```

Finally, to apply PACo:

```
PACo.fit <- PACo.dV(N.D, CP.D, NCP)
NCP.proc <- procrustes(PACo.fit$H.PCo, PACo.fit$P.PCo)
```

The following syntax computes the residual sum of squares m^2_{XY} and randomizes the 'o'-/'n'-association matrix to determine, whether the probability p under H_o ('similarity between trees not higher than expected by chance', see main text) is rejected. The user must set a number of random permutations of the organelle-/host nucleus-matrix. Although we employed 100,000 in all analyses, a number ≤ 10,000 should be sufficient to obtain comparable results.

```
m2.obs <- NCP.proc$ss
N.perm = 10000
P.value = 0
set.seed(2)
for (n in c(1:N.perm))
{
  if (NLinks <= nrow(NCP) | NLinks <= ncol(NCP))
  {  flag2 <- TRUE
     while (flag2 == TRUE)  {
       NCP.perm <- t(apply(NCP,1,sample))
       if(any(colSums(NCP.perm) == NLinks)) flag2 <- TRUE else
flag2 <- FALSE
     }
  } else { NCP.perm <- t(apply(NCP,1,sample))}
  PACo.perm <- PACo.dV(N.D, CP.D, NCP.perm)
  m2.perm <- procrustes(PACo.perm$H.PCo, PACo.perm$P.PCo)$ss
  if (m2.perm <= m2.obs)
```

```
  {P.value = P.value + 1}
}
P.value <- P.value/N.perm

cat(" The observed m2 is ", m2.obs, "\n", "P-value = ", P.value,
" based on ", N.perm," permutations.")
```

Note that `set.seed(2)` sets a reproducible set of test permutations. Changing the integer value will produce a different set, but should not change the *p* value substantially. R will print out the *p* value and m^2_{XY}:

```
The observed m2 is 0.4655883
P-value = 0.0001 based on 1000 permutations.
```

Thus, the significance value at which H_0 is rejected is 0.0001. This shows that, despite the presence of outliers in the phylogenies, organellar and nuclear data sets in *Satyrium* reflect cophylogeny to some degree.

Detecting outlier associations

The contribution (e_i^2) to the global squared residual value (m^2_{XY}) and the $pfl2_i$ (see methods) of each association, using phylograms and unit branch length trees is computed using:

```
lapply(1:length(treeH), D.wrapper)
```

At execution, tables containing e_i^2 and $pfl2_i$ values for each association (for both PACo and ParaFit analyses using phylograms and unit branch length trees) will be generated and saved in your working directory (files `PACo_res_add.txt`, `PACo_res_top.txt`, `PFL2_add.txt` and `PFL2_top.txt`). These tables are required by the pipeline (see below) to spot outlier sequences onto the phylogenies and can be loaded onto the workspace:

```
colnamesPACo <- read.table(file="colnamesPACo.txt", header=TRUE)
colnamesPACo <- colnames(colnamesPACo)

pac.add <- read.table(file="PACo_res_add.txt", header=FALSE,
col.names=colnamesPACo)
pac.top <- read.table(file="PACo_res_top.txt", header=FALSE,
col.names=colnamesPACo)
```

```
pf2.add <- read.table(file="PFL2_add.txt", header=FALSE,
col.names=colnamesPACo)
pf2.top <- read.table(file="PFL2_top.txt", header=FALSE,
col.names=colnamesPACo)
```

Next, outlier associations will be spotted by the pipeline using a threshold value (1/N). The following syntax will transform the e_i^2's into ε_i^2's obtained from either phylograms or unit branch length trees and will compute their respective median. Given the asymmetric distribution of the ε_i^2's, the median value was preferred over the mean as central tendency estimate:

```
m2A <- apply(pac.add, 1, sum)
pac.norm.add <- pac.add/m2A

m2T <- apply(pac.top, 1, sum)
pac.norm.top <- pac.top/m2T
```

To plot the median ε_i^2 and its 95% empirical confidence intervals obtained from sequences in phylograms and unit branch lengths, and to spot outlier taxa according to the threshold value (1/N), use the following script:

```
op <- par(oma=c(3,2,1,1))
par (mfrow=c(1,1),mar = c(4,4,1,1))

mA <- apply(pac.norm.add, 2, median)
uCI.A <- apply(pac.norm.add, 2, quantile, probs = 0.975)
lCI.A <- apply(pac.norm.add, 2, quantile, probs = 0.025)
cols <- c("lightgreen", "mistyrose")[(mA > 1/NLinks) + 1]
barplot2(mA, main = "PAco squared residuals - additive trees",
xlab="Association", ylab="Normalized PACo sqr. residuals",
        cex.axis=0.5, col=cols, border="lightgrey",
names.arg=colnamesPACo, las=2, cex.names=0.5, plot.ci=T,
ci.l=lCI.A, ci.u=uCI.A, ci.color="blue")
abline(h=1/NLinks, col="red")

mA <- apply(pac.norm.top, 2, median)
uCI.A <- apply(pac.norm.top, 2, quantile, probs = 0.975)
lCI.A <- apply(pac.norm.top, 2, quantile, probs = 0.025)
cols <- c("lightgreen", "mistyrose")[(mA > 1/NLinks) + 1]
barplot2(mA, main = "PAco squared residuals - unit branch length
trees", xlab="Association", ylab="Normalized PACo
sqr.residuals", cex.axis=0.5, col=cols, border="lightgrey",
```

```
names.arg=colnamesPACo, las=2, cex.names=0.5, plot.ci=T,
ci.l=lCI.A, ci.u=uCI.A, ci.color="blue")
abline(h=1/NLinks, col="red")
```

Two plots (Fig. S3, data with ε_i^2's obtained from unit branch length trees not shown) of all squared residual values determined from each 'o'-/'n'-association, and obtained from phylograms and unit branch lengths as well, will be plotted, respectively. Associations with ε_i^2's scores above the red line (i.e., 1/N threshold value) represent putative outlier sequences especially, if the lower bound of the associated 95% confidence interval is above the threshold. In the working example of *Satyrium*, 15 'o'-/'n'-associations were retrieved as outlier (Fig. S3). Eleven of such links presented indeed contrasting phylogenetic positions on chloroplast and nuclear trees (red bars in Fig. S3). All outlier associations detected by PACo as potentially outliers are shown in Figure S4. Names in red correspond to associations retrieved by PACo that are true outliers, whereas names in black are associations identified by PACo as potential outliers, even though they did not recover conflicting phylogenetic positions. In our simulations and real data set analyses *pfl2*$_i$ yielded suboptimal results, but the user may also wish to plot the *pfl2*$_i$'s for comparative purposes:

```
mA <- apply(pf2.add, 2, median)
uCI.A <- apply(pf2.add, 2, quantile, probs = 0.975)
lCI.A <- apply(pf2.add, 2, quantile, probs = 0.025)
cols <- c("lightgreen", "mistyrose")[(mA > 0) + 1]
barplot2(mA, main = "pfl2 statistic - additive trees",
xlab="Association", ylab="Normalized PACo sqr. residuals",
          cex.axis=0.5,       col=cols,       border="lightgrey",
names.arg=colnamesPACo,    las=2,    cex.names=0.5,    plot.ci=T,
ci.l=lCI.A,
          ci.u=uCI.A, ci.color="blue")
abline(h=0, col="red")

mA <- apply(pf2.top, 2,  median)
uCI.A <- apply(pf2.top, 2, quantile, probs = 0.975)
lCI.A <- apply(pf2.top, 2, quantile, probs = 0.025)
cols <- c("lightgreen", "mistyrose")[(mA > 0) + 1]
barplot2(mA, main = "pfl2 statistic - unit branch length trees",
xlab="Association", ylab="Normalized PACo sqr. residuals",
cex.axis=0.5, col=cols, border="lightgrey",
names.arg=colnamesPACo, las=2, cex.names=0.5, plot.ci=T,
ci.l=lCI.A, ci.u=uCI.A, ci.color="blue")
abline(h=0, col="red")
```

Validating classifications of outlier and congruent terminals with PAM

Cluster analysis using the Partition Around Medoids (PAM) algorithm (Kaufman and Rousseeuw 1990) allows the end-user to determine the extent of properly classified associations into outlier or congruent OTUs in relation to the total number of OTUs and the proportion of outlier/congruent OTUs. Our pipeline offers two alternatives to carry out clustering analyses, namely 1) using median ε_i^2 and $pfl2_i$ values combined and 2) using median ε_i^2's alone. Our simulations and real data set analyses show that the latter strategy yields stronger cluster structures, but comparison between the two approaches can still be useful to reveal doubtful associations. Clustering starts by standardizing both statistics (ε_i^2 and $pfl2_i$):

```
sum.pac.add <- apply(pac.add, 1, sum)
pac.add <- pac.add/sum.pac.add - 1/NLinks
sum.pac.top <- apply(pac.top, 1, sum)
pac.top <- pac.top/sum.pac.top - 1/NLinks

im.paco.add <- apply(pac.add, 2, median)
im.paco.top <- apply(pac.top, 2, median)
im.pf2.add <- apply(pf2.add, 2, median)
im.pf2.top <- apply(pf2.top, 2, median)

x.paco.add <- mean(im.paco.add) ; x.pf2.add <- mean(im.pf2.add)
sd.paco.add<- sd(im.paco.add)  ; sd.pf2.add <- sd(im.pf2.add)
im.paco.stadd <- (x.paco.add - im.paco.add)/sd.paco.add
im.pf2.stadd <- (x.pf2.add - im.pf2.add)/sd.pf2.add
metrics.stadd <- data.frame(im.paco.stadd, im.pf2.stadd)

x.paco.top <- mean(im.paco.top) ; x.pf2.top <- mean(im.pf2.top)
sd.paco.top <- sd(im.paco.top)  ; sd.pf2.top <- sd(im.pf2.top)
im.paco.sttop <- (x.paco.top - im.paco.top)/sd.paco.top
im.pf2.sttop <- (x.pf2.top - im.pf2.top)/sd.pf2.top
metrics.sttop <- data.frame(im.paco.sttop, im.pf2.sttop)
```

The user must specify the number of clusters (*k*). Initially, one should set *k*=2, as PAM is expected to separate the 'o'-/'n'-associations into non-conflicting and outlier. However, in some situations *pfl2* tends to split non-conflicting associations into two unnatural clusters, and *k* has to be set to 3 in order to retrieve the group of outlier associations.

```
nclust = my k
```

To apply clustering analysis using PACo in combination with *pfl2* with both phylograms and unit branch length trees use the following commands:

```
par (mfrow=c(2,1))
K.PAM <- pam(metrics.stadd, nclust, diss=FALSE)
plot(im.paco.add,im.pf2.add,
col=c("red","blue")[K.PAM$clustering])
title(main=list("PACo-Parafit - additive trees", cex=0.8))
SPaPf.add <- silhouette(K.PAM)
cat(summary(SPaPf.add)$avg.width)
SPaPf.add  <- summary(SPaPf.add)$avg.width
cat("\n")

K.PAM <- pam(metrics.sttop, nclust, diss=FALSE)
plot(im.paco.top,im.pf2.top,
col=c("red","blue")[K.PAM$clustering])
title(main=list("PACo-pf2 - unit branch length trees", cex=0.8))
SPaPf.top <- silhouette(K.PAM)
cat(summary(SPaPf.top)$avg.width)
SPaPf.top  <- summary(SPaPf.top)$avg.width
cat("\n")
```

In contrast, the end-user might want to apply clustering analysis using solely PACo with phylograms and unit branch length trees:

```
K.PAM <- pam(metrics.stadd[1], nclust, diss=FALSE)
plot(im.paco.add,im.pf2.add,
col=c("red","blue")[K.PAM$clustering])
title(main=list("PACo + additive trees", cex=0.8))
SPa.add <- silhouette(K.PAM)
cat(summary(SPa.add)$avg.width)
SPa.add <- summary(SPa.add)$avg.width
cat("\n")

K.PAM <- pam(metrics.sttop[1], nclust, diss=FALSE)
plot(im.paco.top,im.pf2.top,
col=c("red","blue")[K.PAM$clustering])
title(main=list("PACo - unit branch length trees", cex=0.8))
SPa.top <- silhouette(K.PAM)
cat(summary(SPa.top)$avg.width)
SPa.top <- summary(SPa.top)$avg.width
```

```
cat("\n")
```

All silhouette values from all clustering analysis on a single table can also be save on disk:

```
Sall <- rbind(SPaPf.add, SPa.add, SPaPf.top, SPa.top)
rownames(Sall) <- c("Silhouette PACo-Parafit additive",
"Silhouette PACo additive", "Silhouette PAco-Parafit unit branch
length", "Silhouette PACo unit branch length ")
write.table(Sall, "Silhouette_values_all.txt")
```

Spotting outlier associations on trees

In order to allow the end-user a better representation of potential outlier associations on trees, our pipeline finally produces a cophylogenetic plot of organellar and nuclear trees with outlier OTUs directly labeled on trees by means of a color scale:

```
op <- par(oma=c(1,1,1,1))
par (mfrow=c(1,2),mar = c(1,1,1,1))

mA <- apply(pac.norm.add, 2, median)
mA[mA > 1/NLinks] <- 1
mA[mA < 1/NLinks] <- 0
mA <- as.data.frame(mA)
out <- mA$mA
names(out) <- NTree$tip.label
out

plotTree(NTree, setEnv = T, offset=0.5, fsize=0.5, lwd=1)
title(main="Nuclear tree of Gene 1 - PACo potential conflicting
associations", font.main=1, cex.main=0.8)
tiplabels(pie = to.matrix(out, sort(unique(out))), piecol =
c("lightgreen", "lightcoral"), cex = 0.5)
legend("bottomleft", c("Congruent", "Conflicting"),
       cex=0.9, pch=16, col=c("lightgreen", "lightcoral"))

plotTree(CPTree, setEnv = T, offset=0.5, fsize=0.5, lwd=1)
title(main="Chloroplast tree of Gene 2 - PACo potential
conflicting associations", font.main=1, cex.main=0.8)
tiplabels(pie = to.matrix(out, sort(unique(out))), piecol =
c("lightgreen", "lightcoral"), cex = 0.5)
```

This script will plot the consensus trees of each data set analyzed, with the corresponding OTUs names. Their individual ε_i^2 scores are color-coded according to their values (conflicting or congruent). The color scale can be bespoke, by replacing the argument `"piecol"` with any alternative allowed by the function. In the working example (results with unit branch length trees not shown), the cophylogenetic plot of the consensus chloroplast and nuclear trees, together with their color-coded ε_i^2 scores (Fig. S5), largely reflects the results observed in the confidence interval plot (Fig. S3). The script also allows to easily spot outlier OTUs in large phylogenies (see Figs S6, S7 for a barplot with PACo squared residual values and plotted simulated trees of 200 OTUs showing outlier associations highlighted by PACo as potential outliers, respectively).

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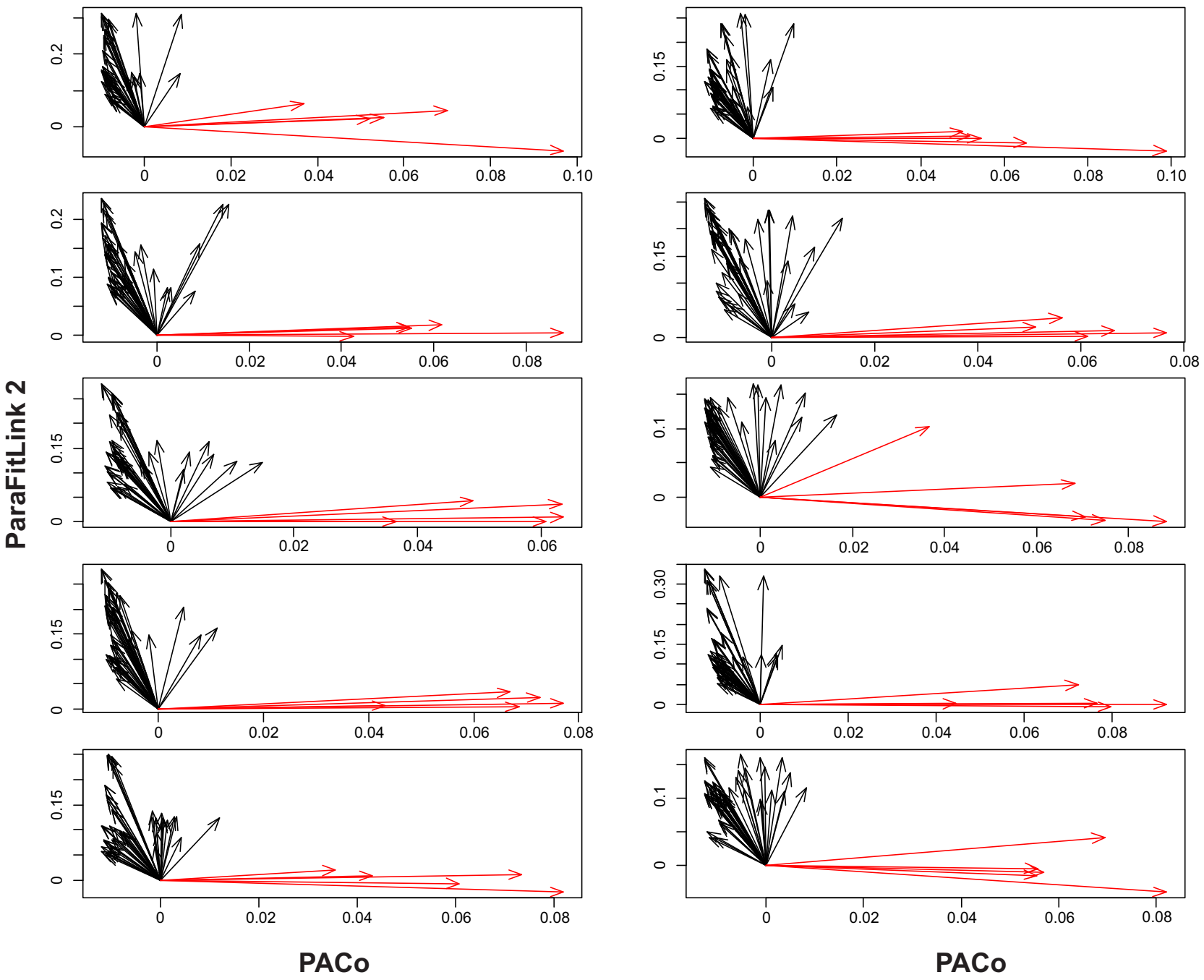
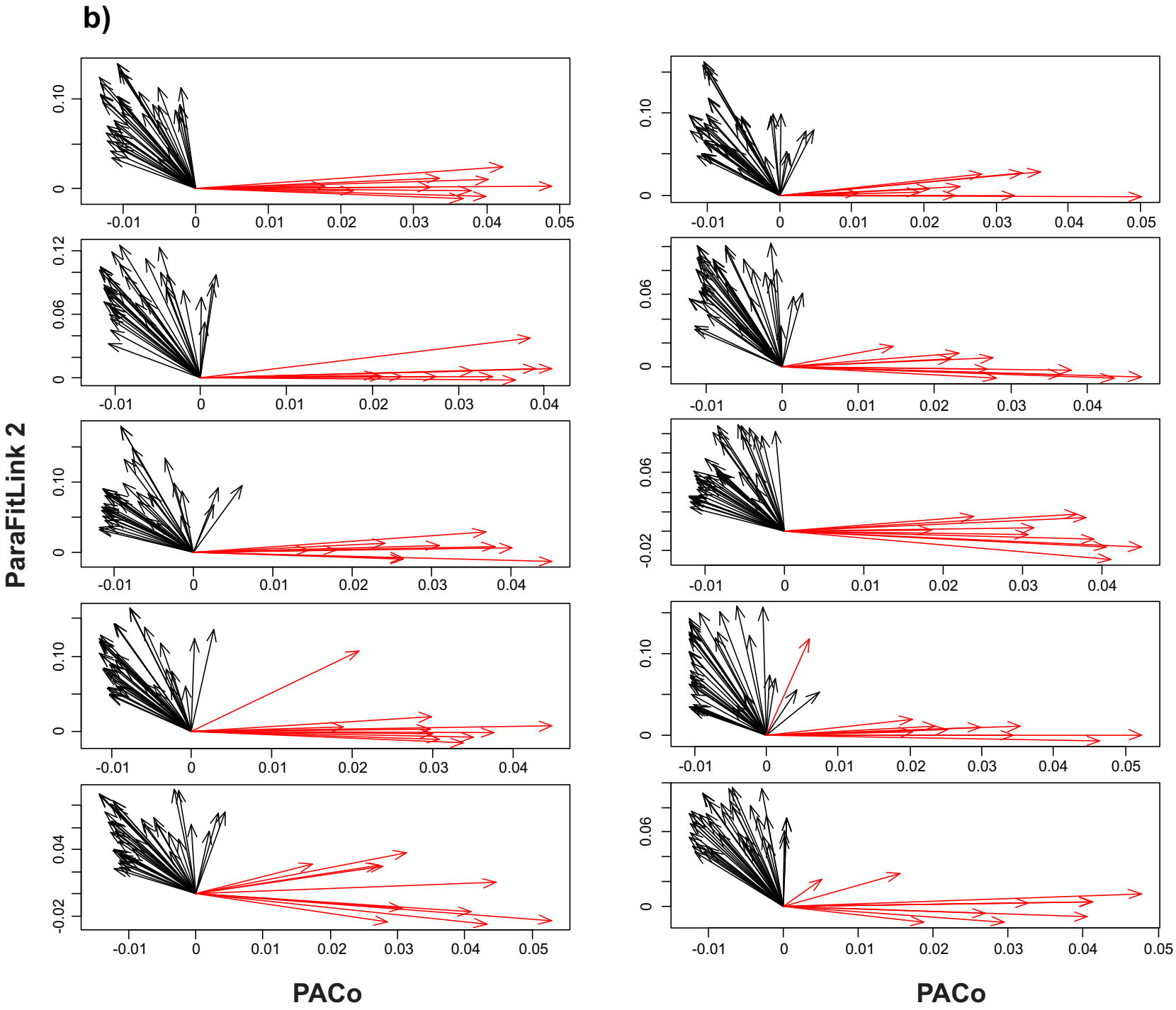
FIGURES

Figure S1. Vector diagrams of squared residual values ε_i^2 and ParaFitLink2 statistic (*pfl2*) obtained by PACo and ParaFit, respectively, using simulated additive trees. Vector magnitude and orientation are related to the topological degree congruence of each 'o'-/'n'-association. Outlier associations are shown in red and non-conflicting in black. Trees with 50 terminals including a) 5 outliers (10%); b) 10 outliers (20%); c) 15 outliers (30%); d) 20 outliers (40%); with 100 terminals including e) 10% outliers; f) 20% outliers; g) 30% outliers; h) 40% outliers; with 200 terminals including i) 10% outliers; j) 20% outliers; k) 30% outliers; l) 40% outliers.

Fig. S1

a)

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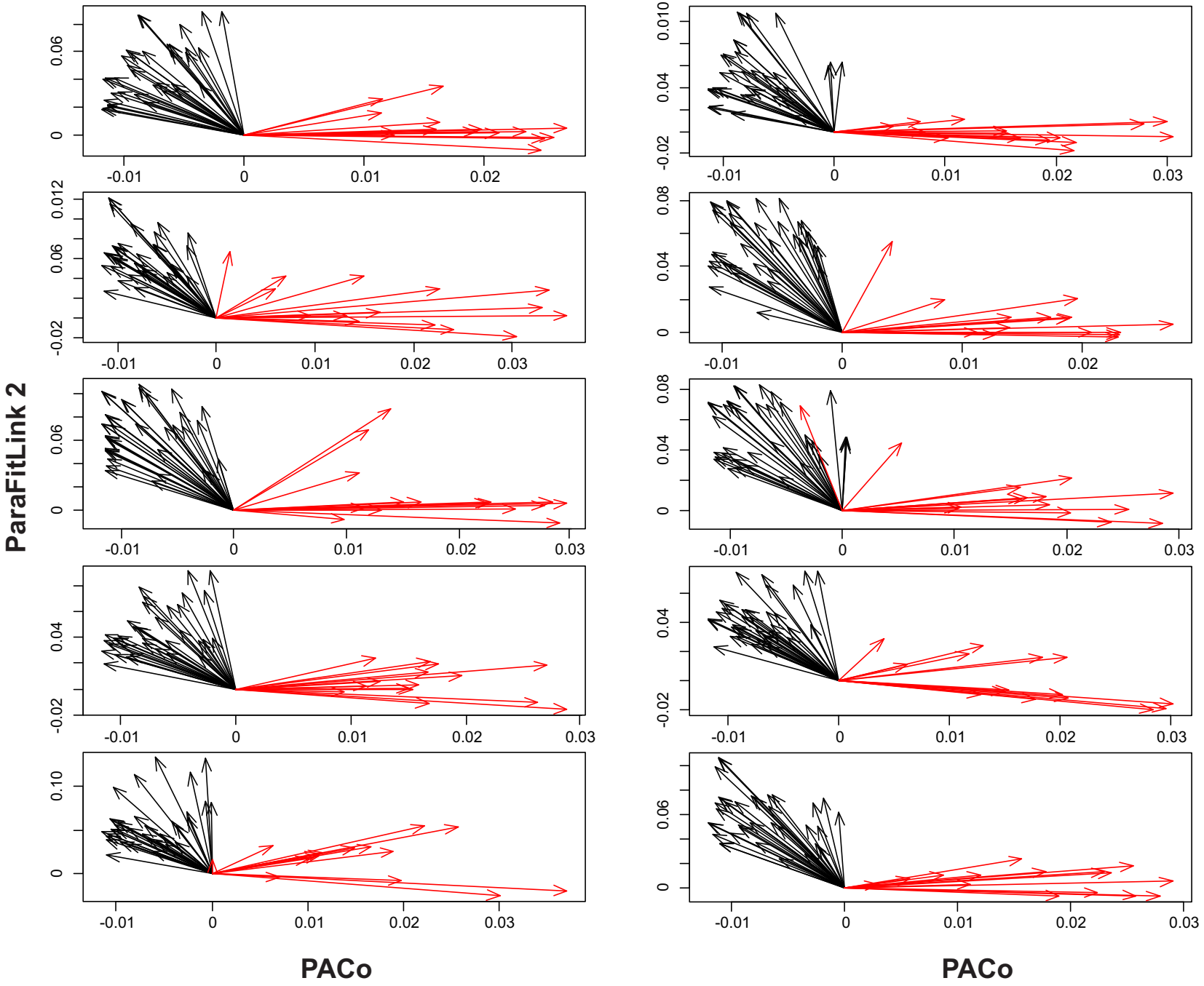
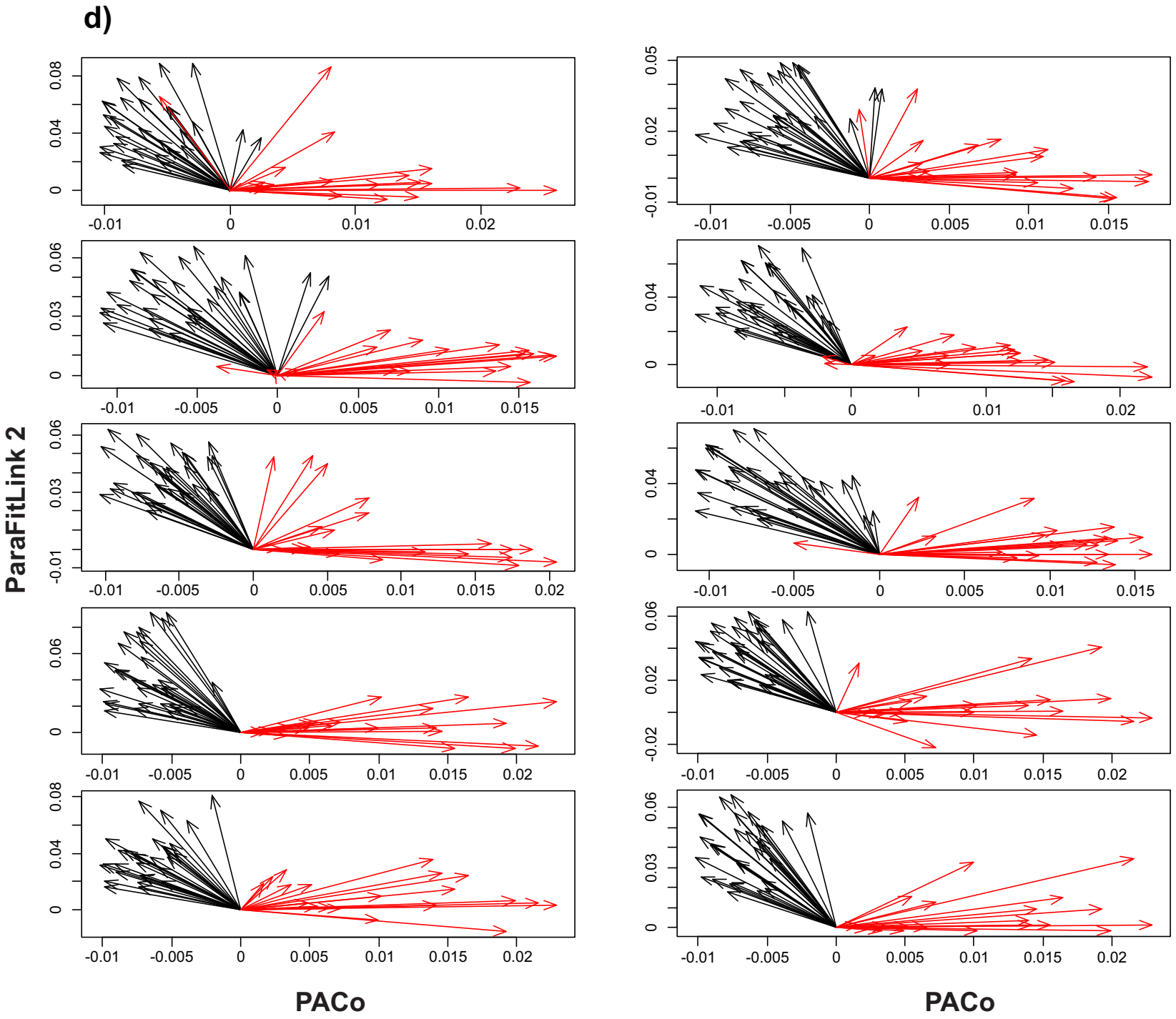
ParaFitLink 2
PACo
PACo

c)

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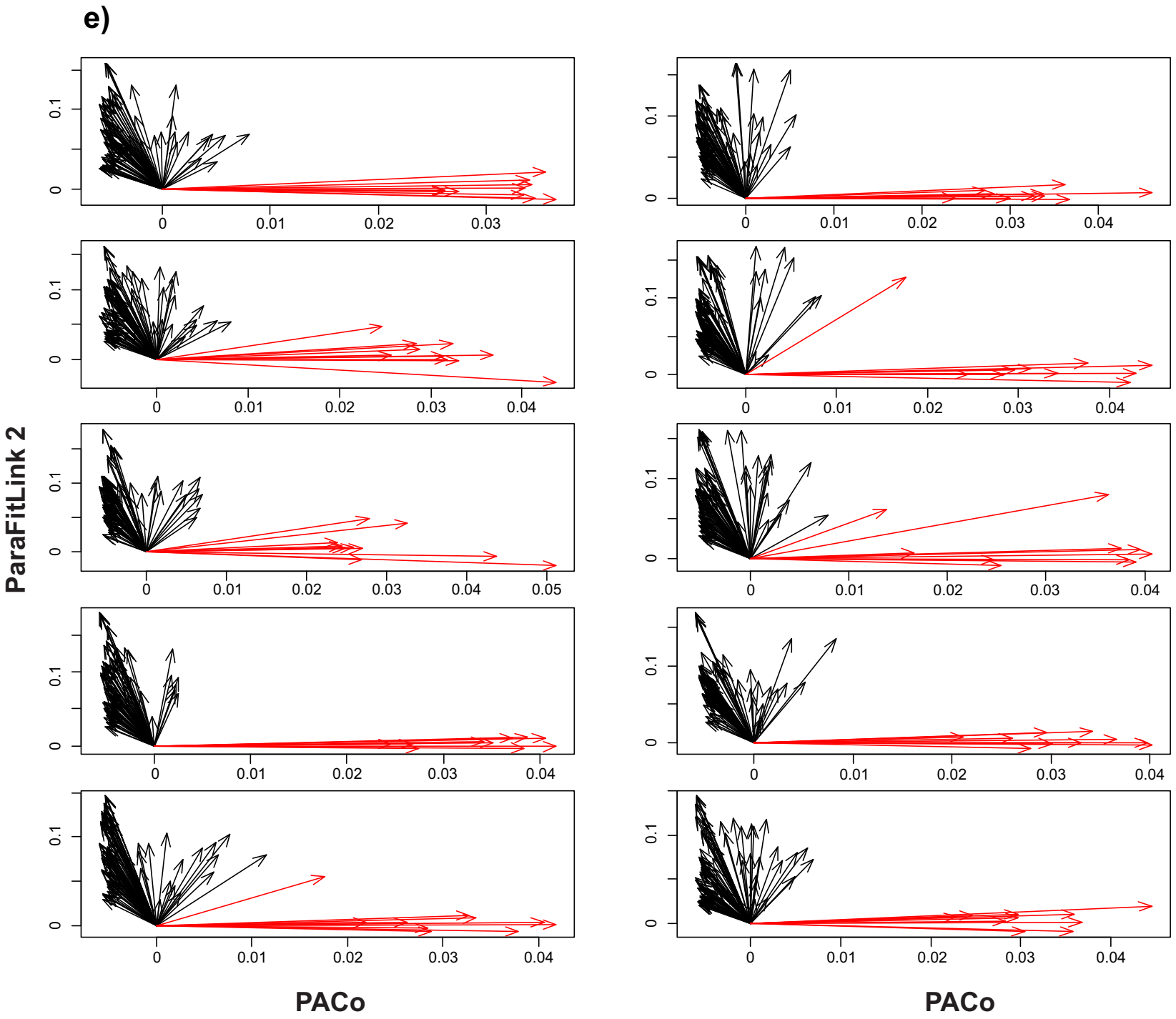
PACo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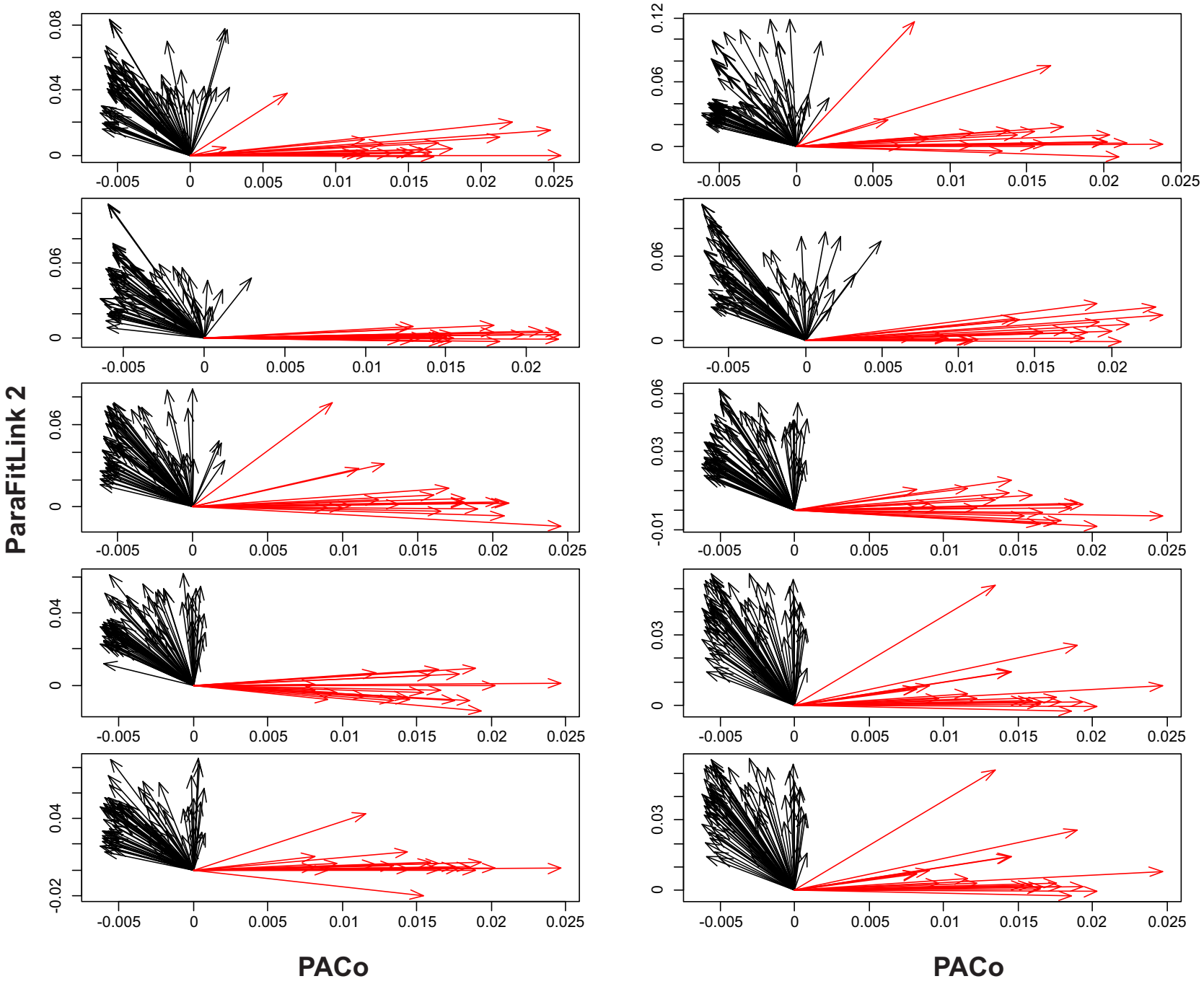
PACo
PACo

f)



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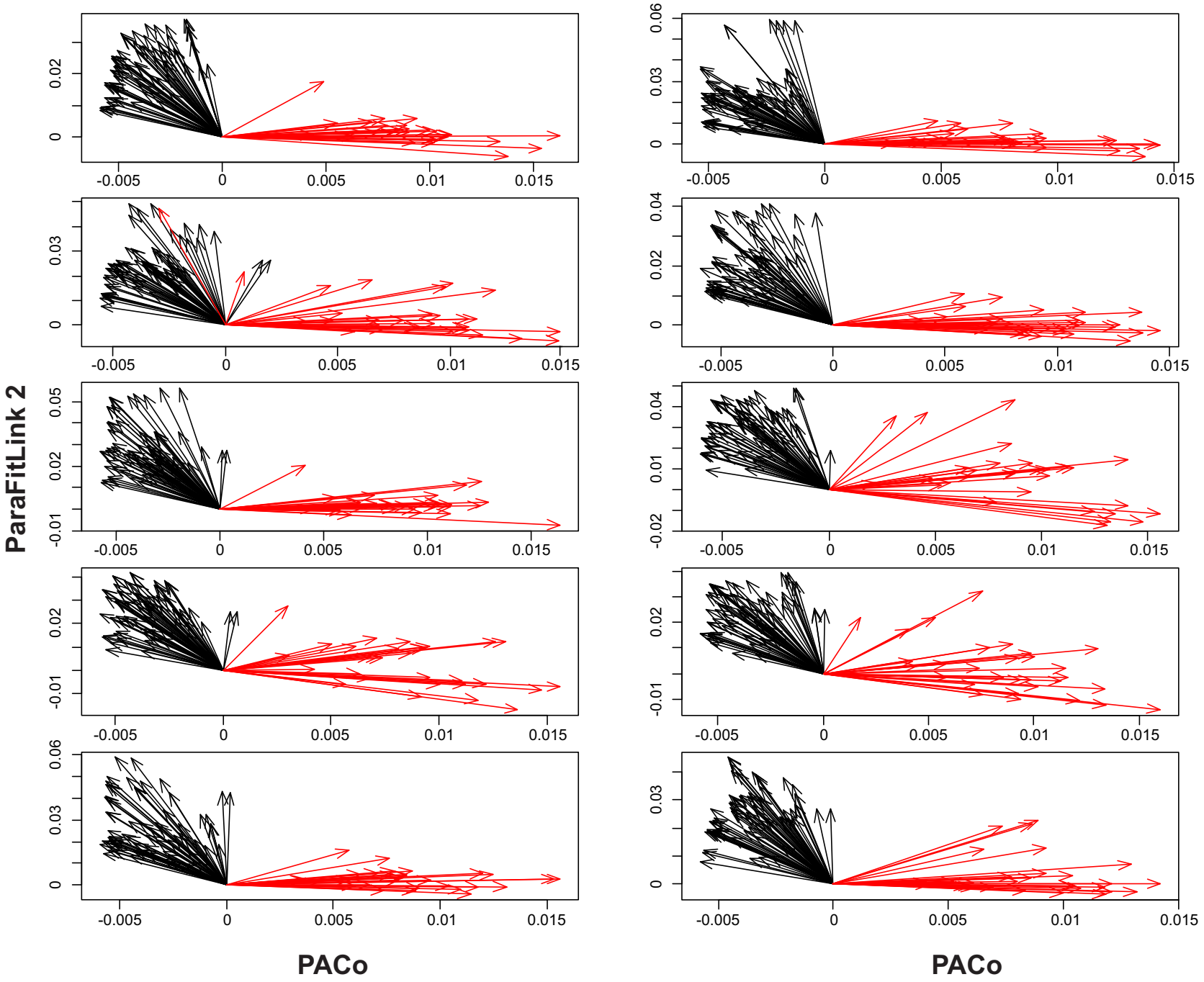

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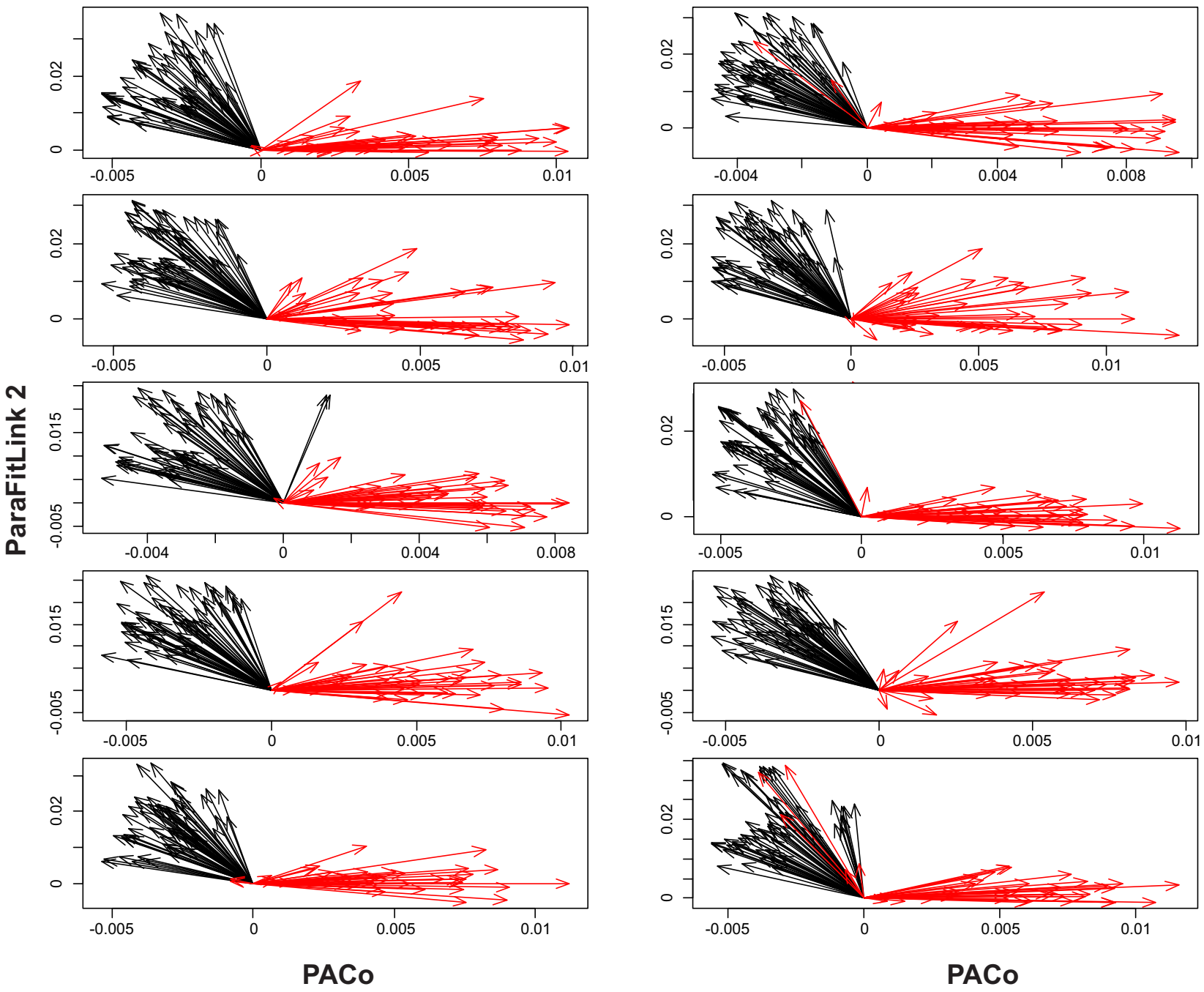

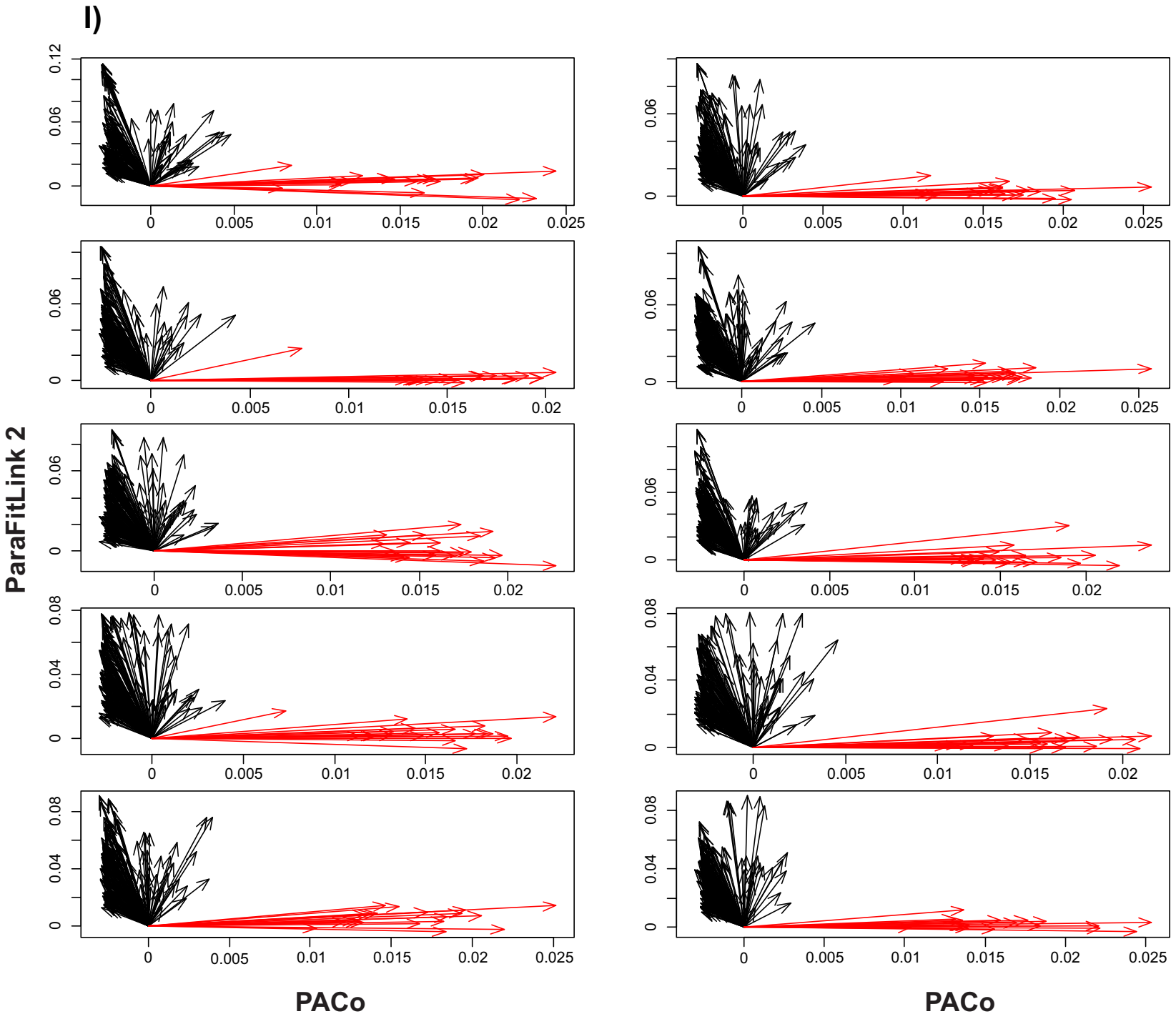
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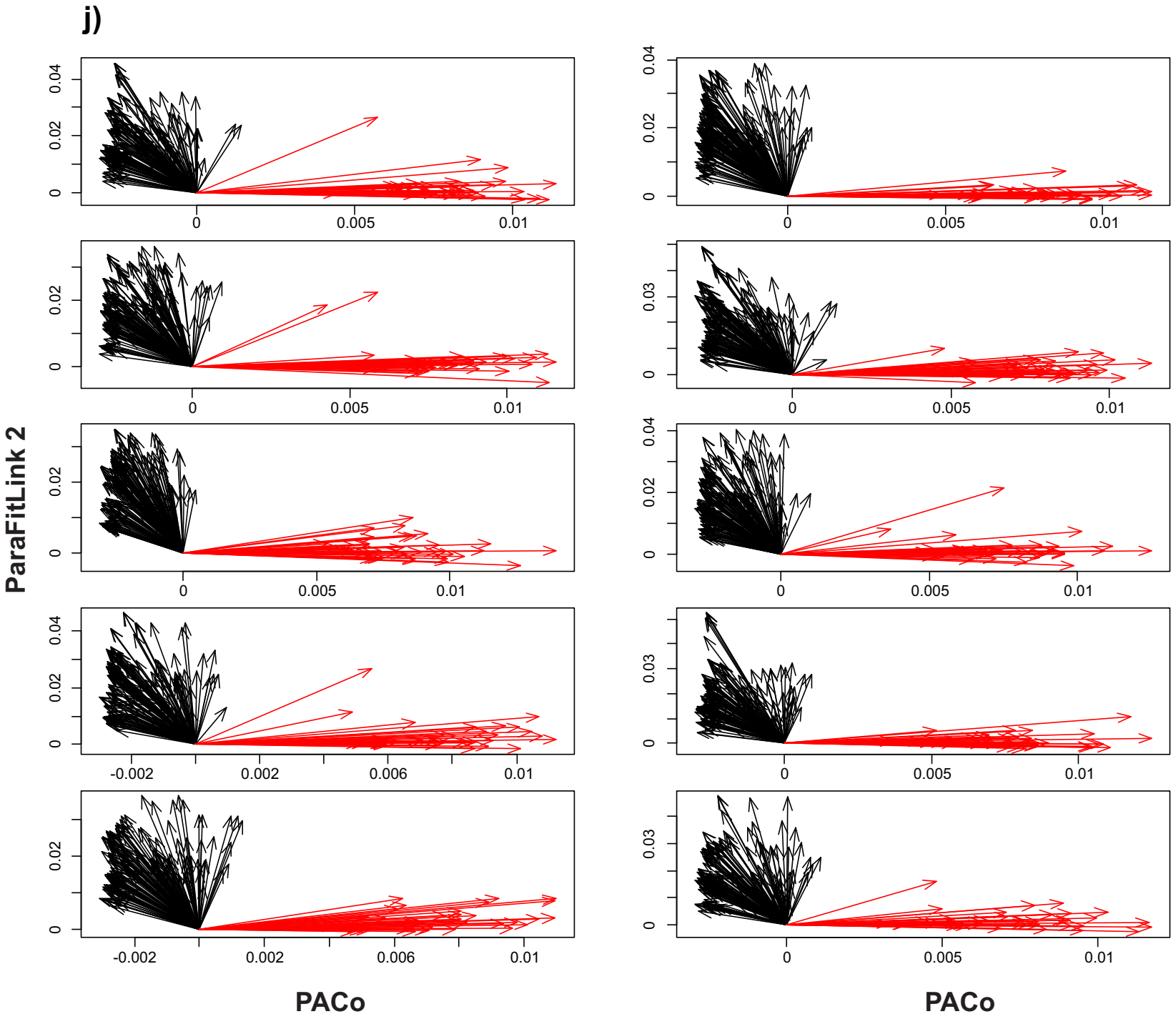
PACo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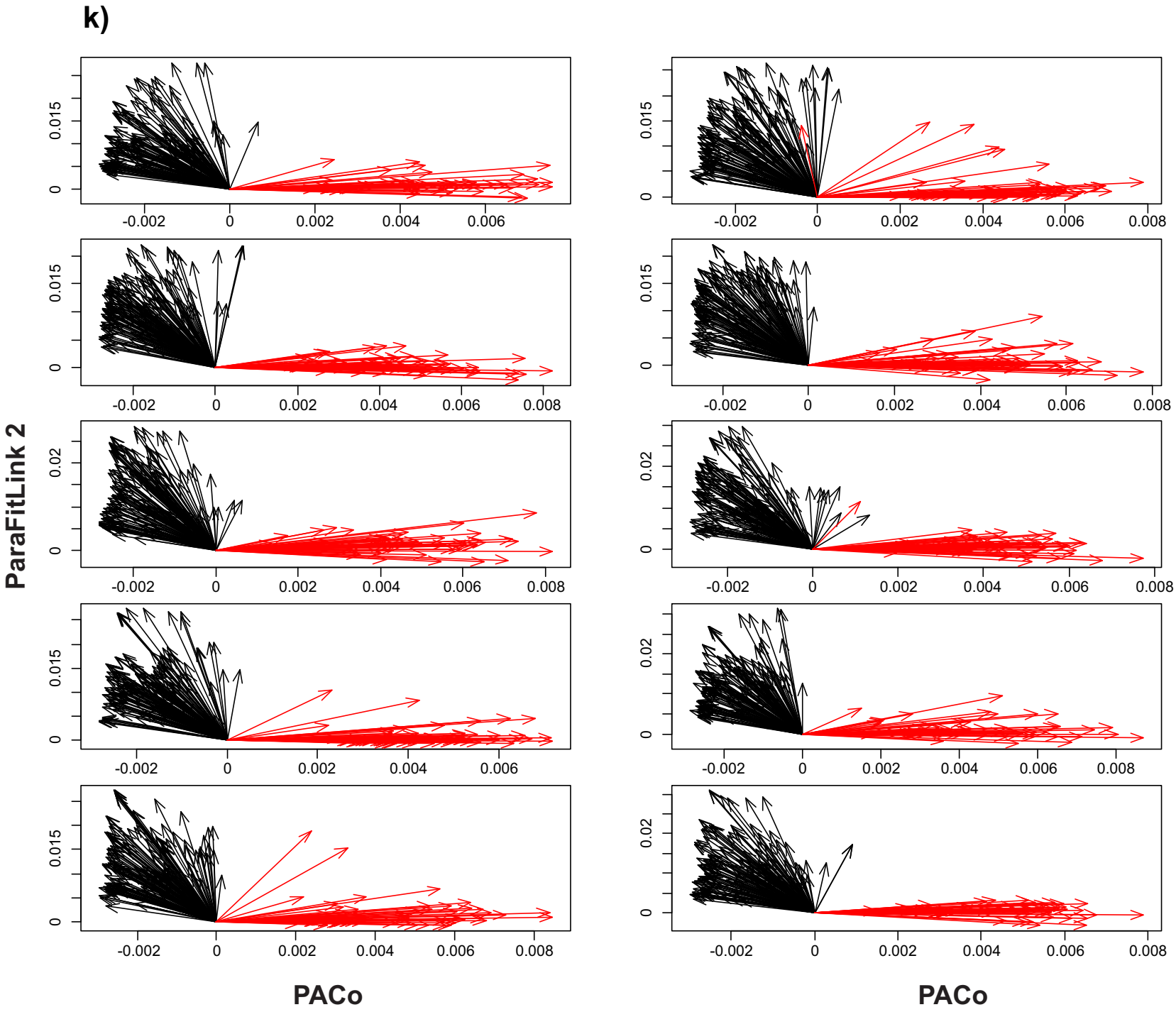
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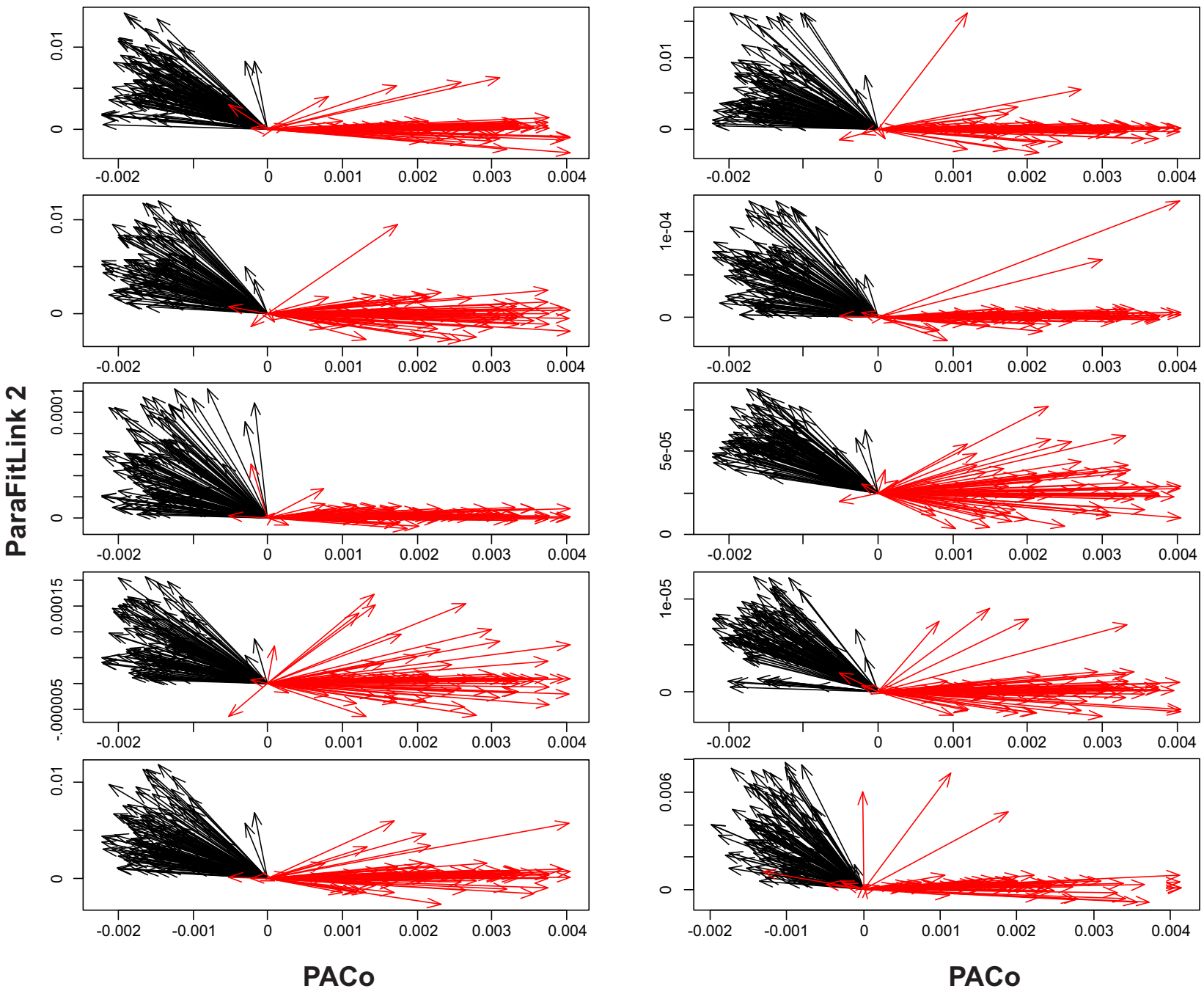
ParaFitLink 2
PACo
PACo

I)


ParaFitLink 2
PACo
PACo
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-0.001
0
0.001
0.002
0.003
0.004
0.01
0
0.0001
-.000005
0.00015
1e-04
5e-05
1e-05
0.006

Figure S2. Vector diagrams of squared residual values ε_i^2 and ParaFitLink2 statistic (*pfl2*) using simulated unit branch length trees. Vector magnitude and orientation are related to the topological degree congruence of each 'o'-/'n'-association. Outlier associations are shown in red, non-conflicting in black. Trees with 50 terminals including a) 5 outliers (10%); b) 10 outliers (20%); c) 15 outliers (30%); d) 20 outliers (40%); with 100 terminals including e) 10% outliers; f) 20% outliers; g) 30% outliers; h) 40% outliers; with 200 terminals including i) 10% outliers; j) 20% outliers; k) 30% outliers; l) 40% outliers.

Fig. S2

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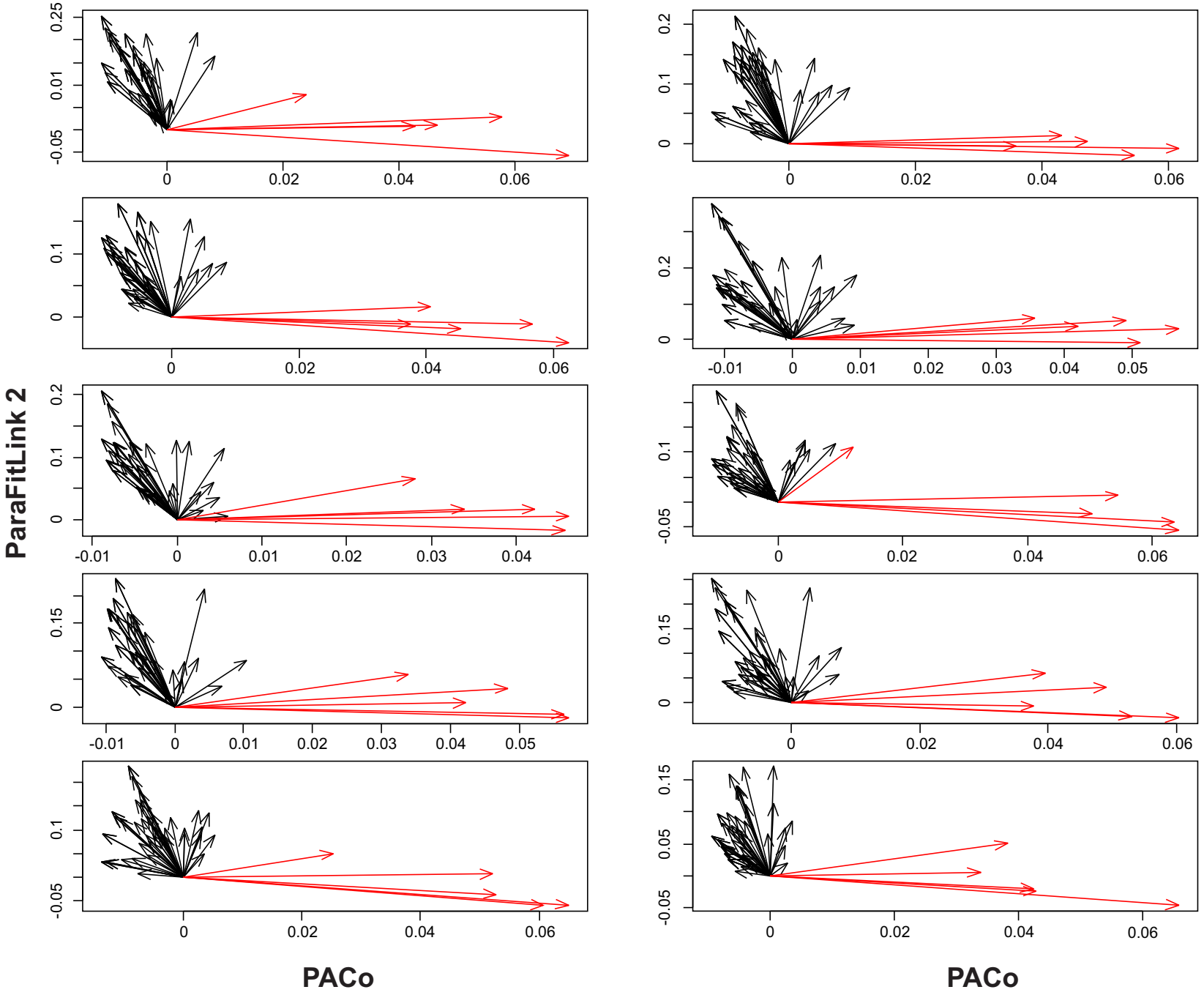

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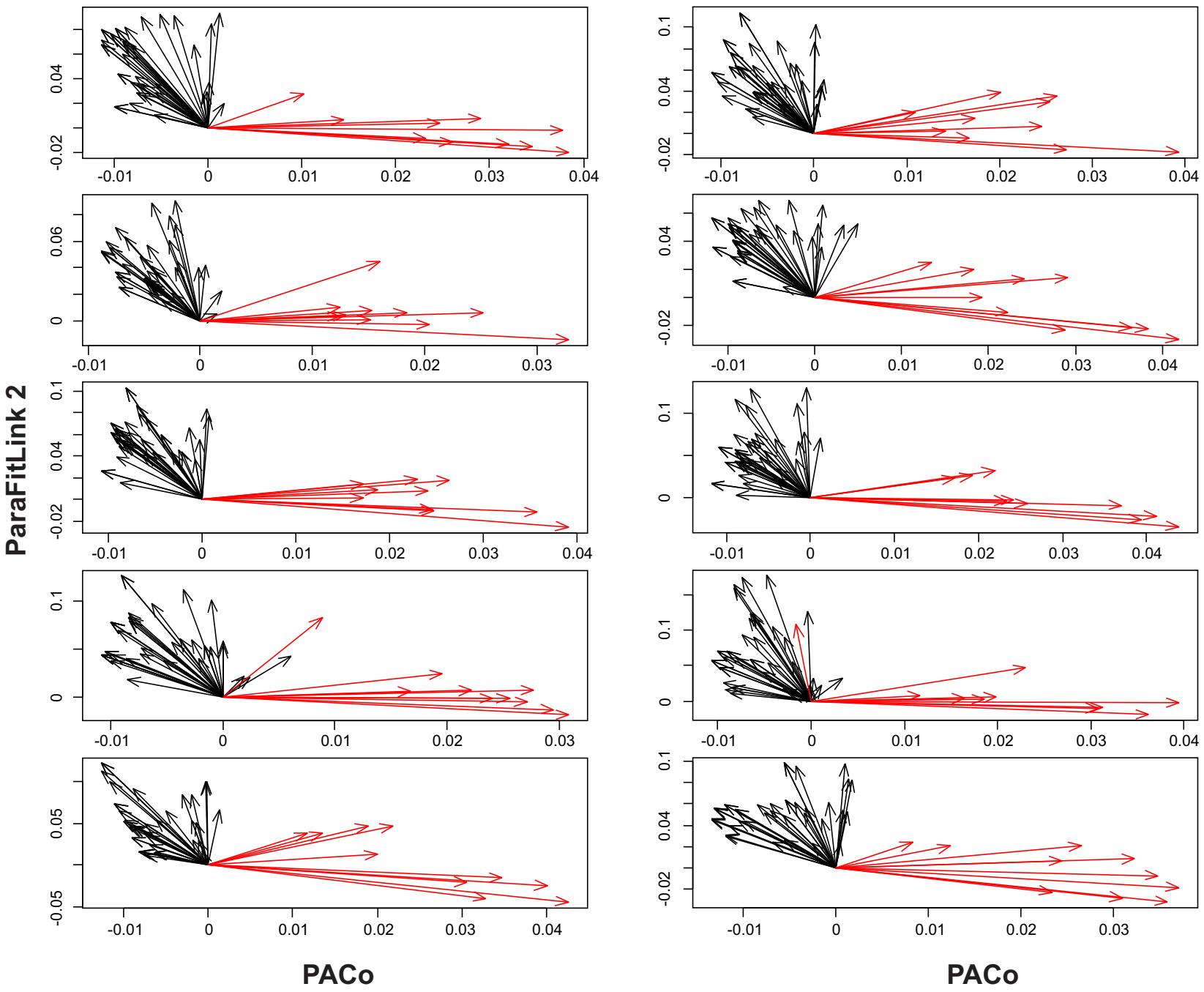
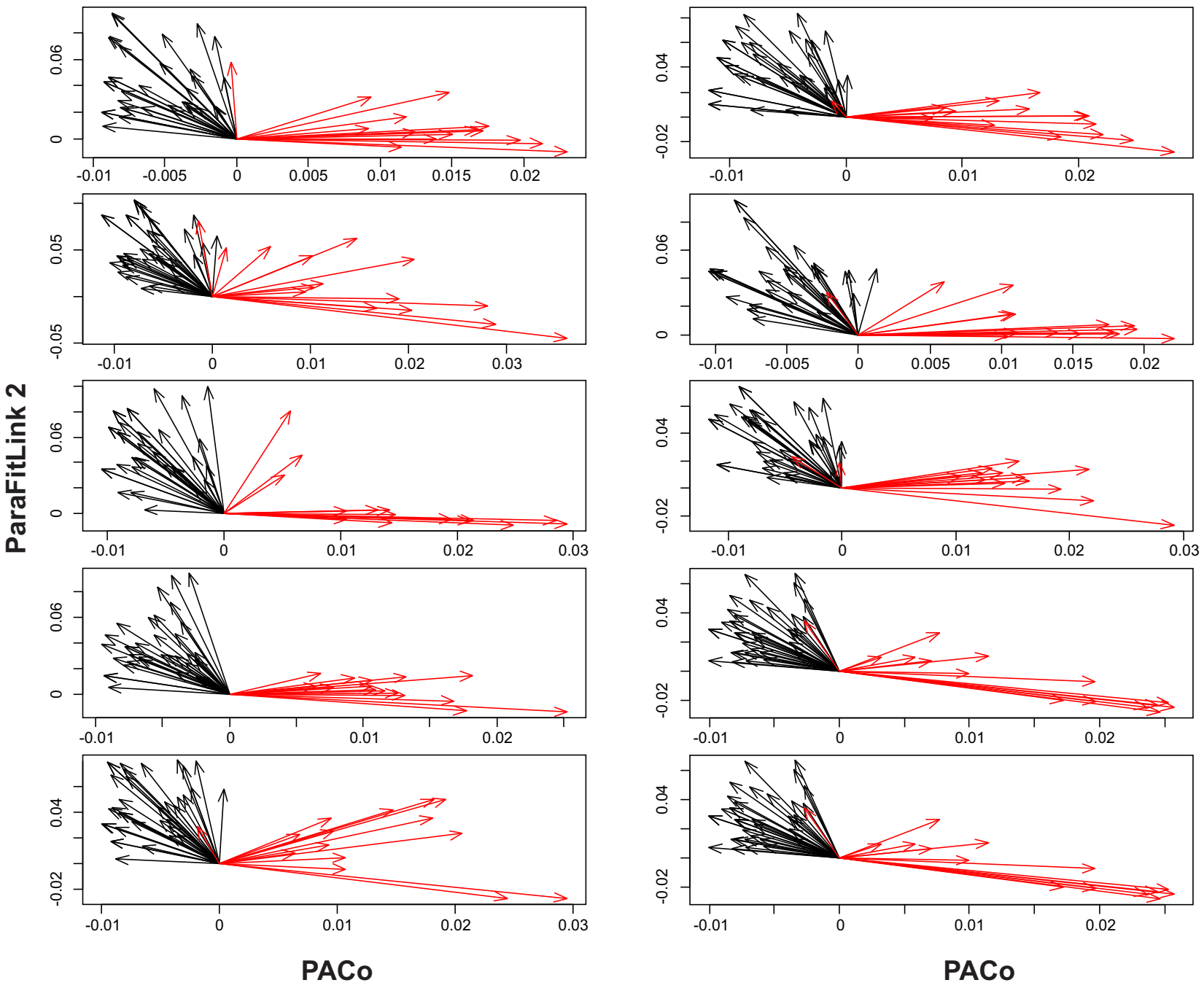


c)



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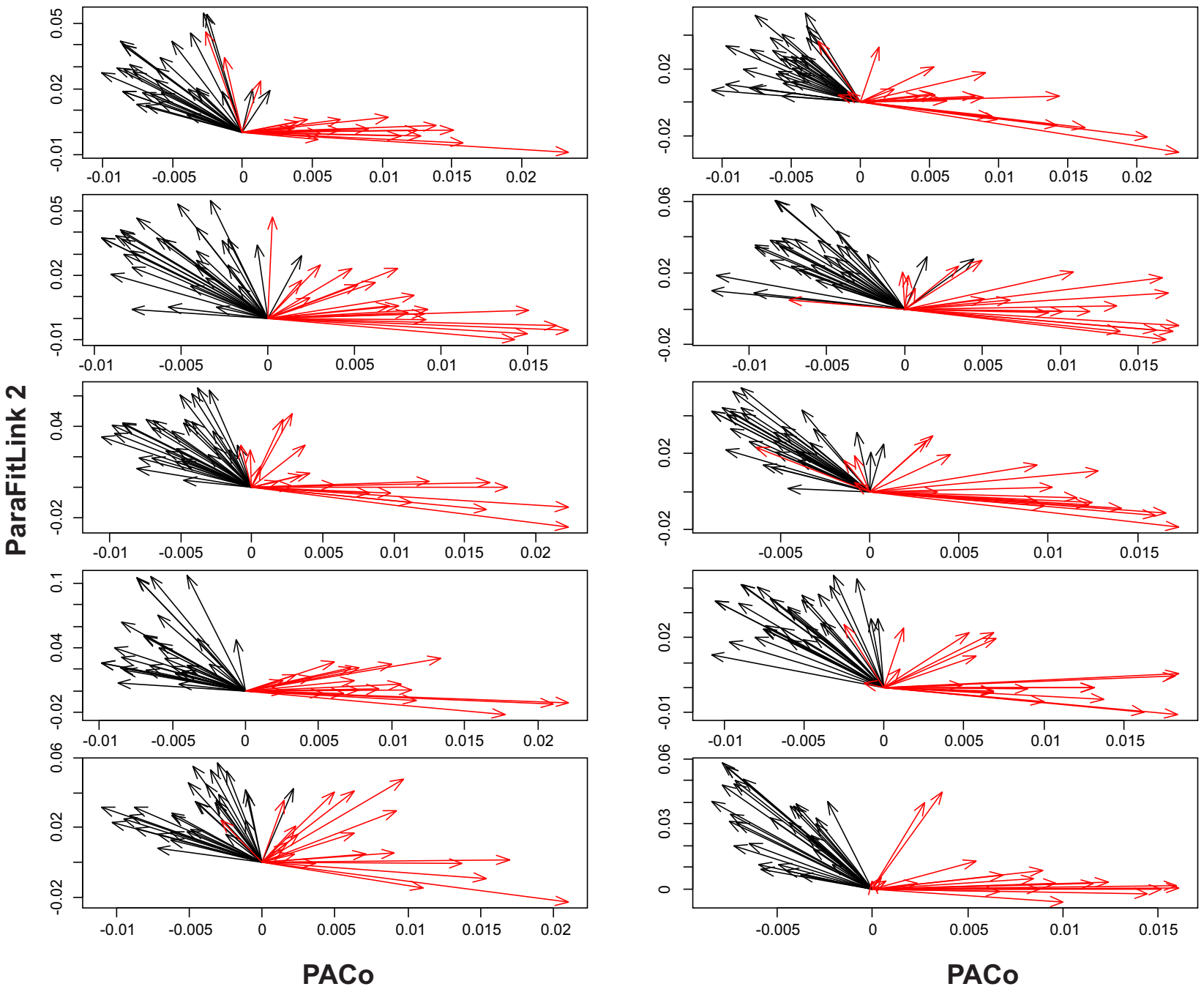

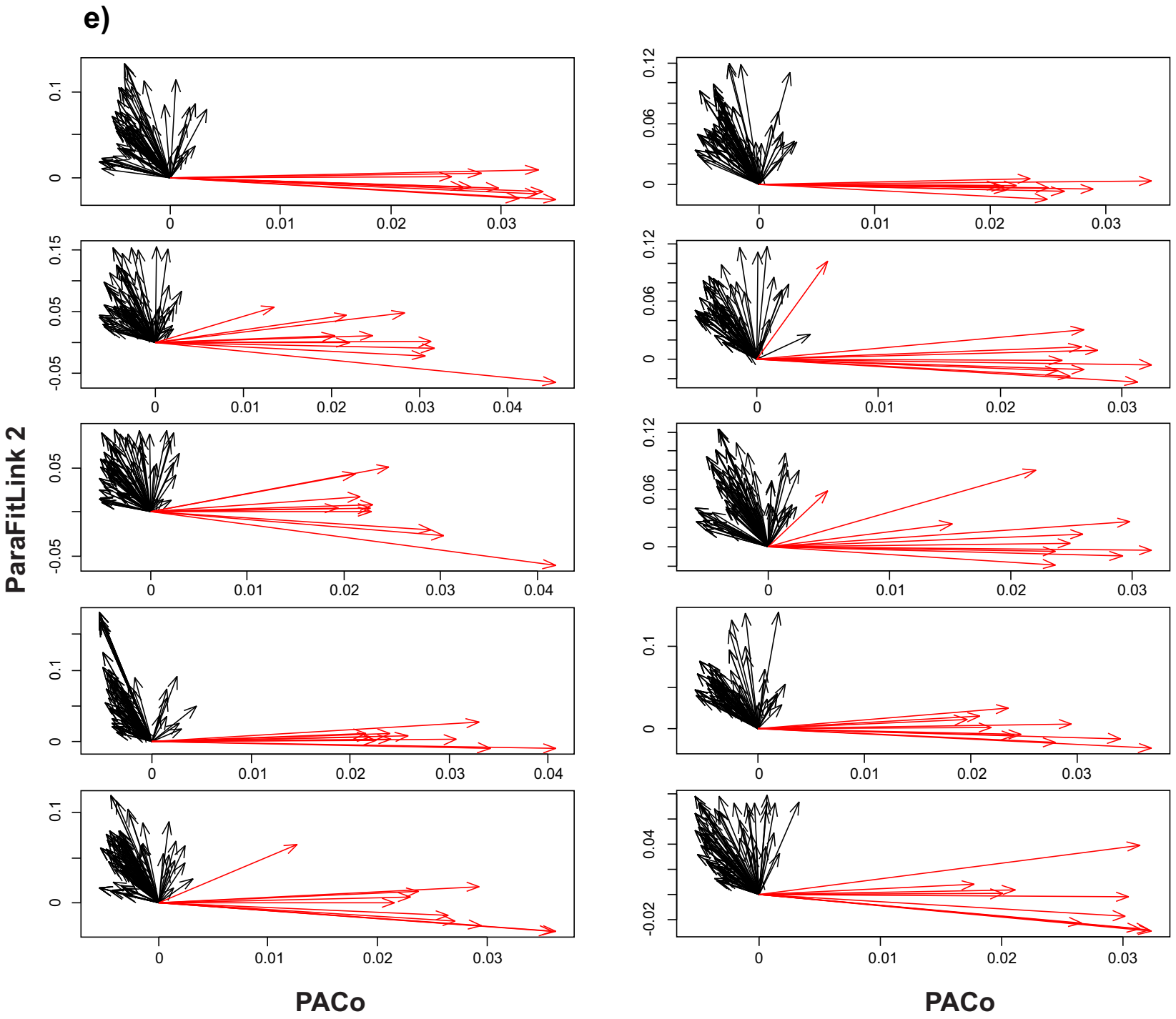
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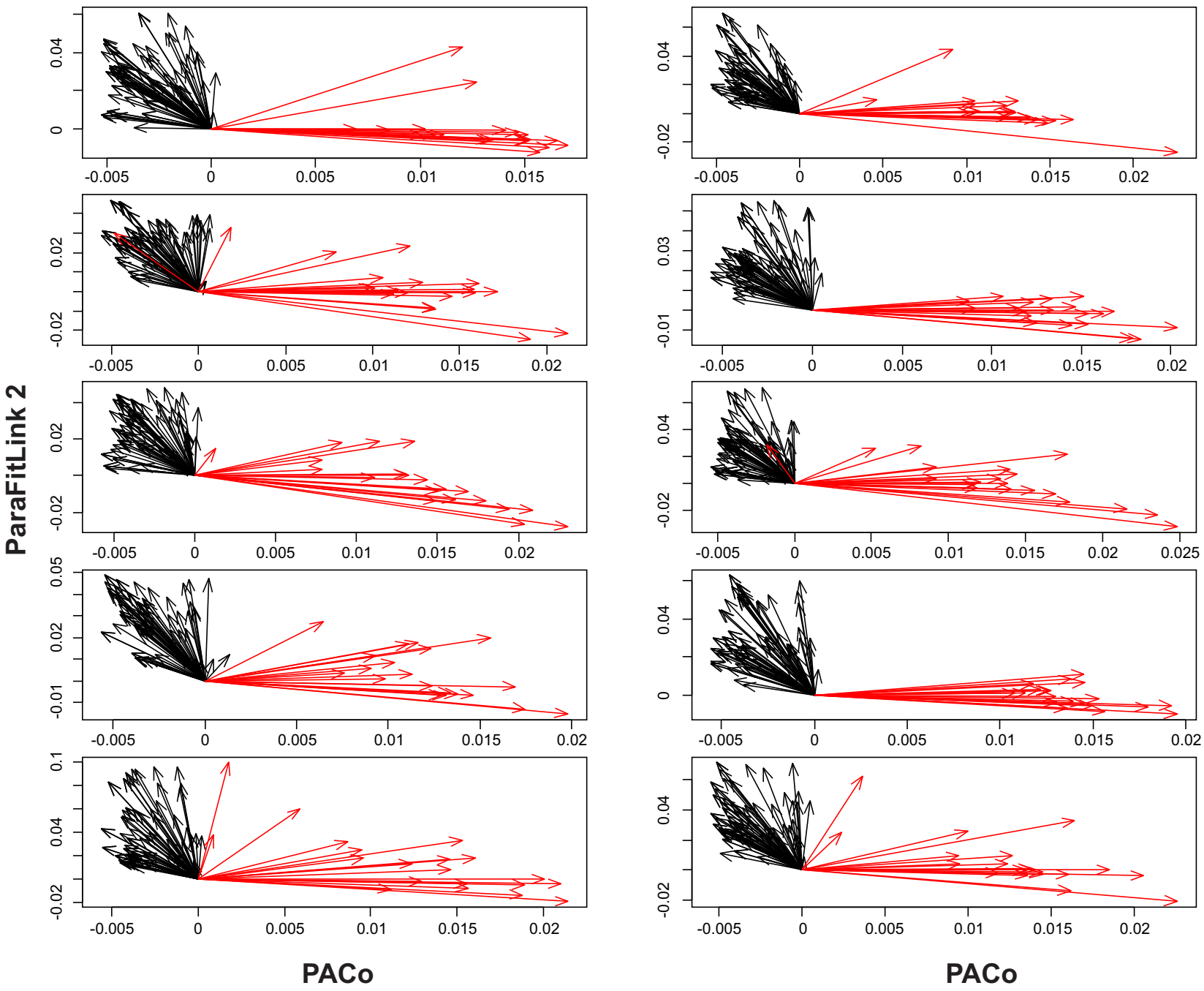
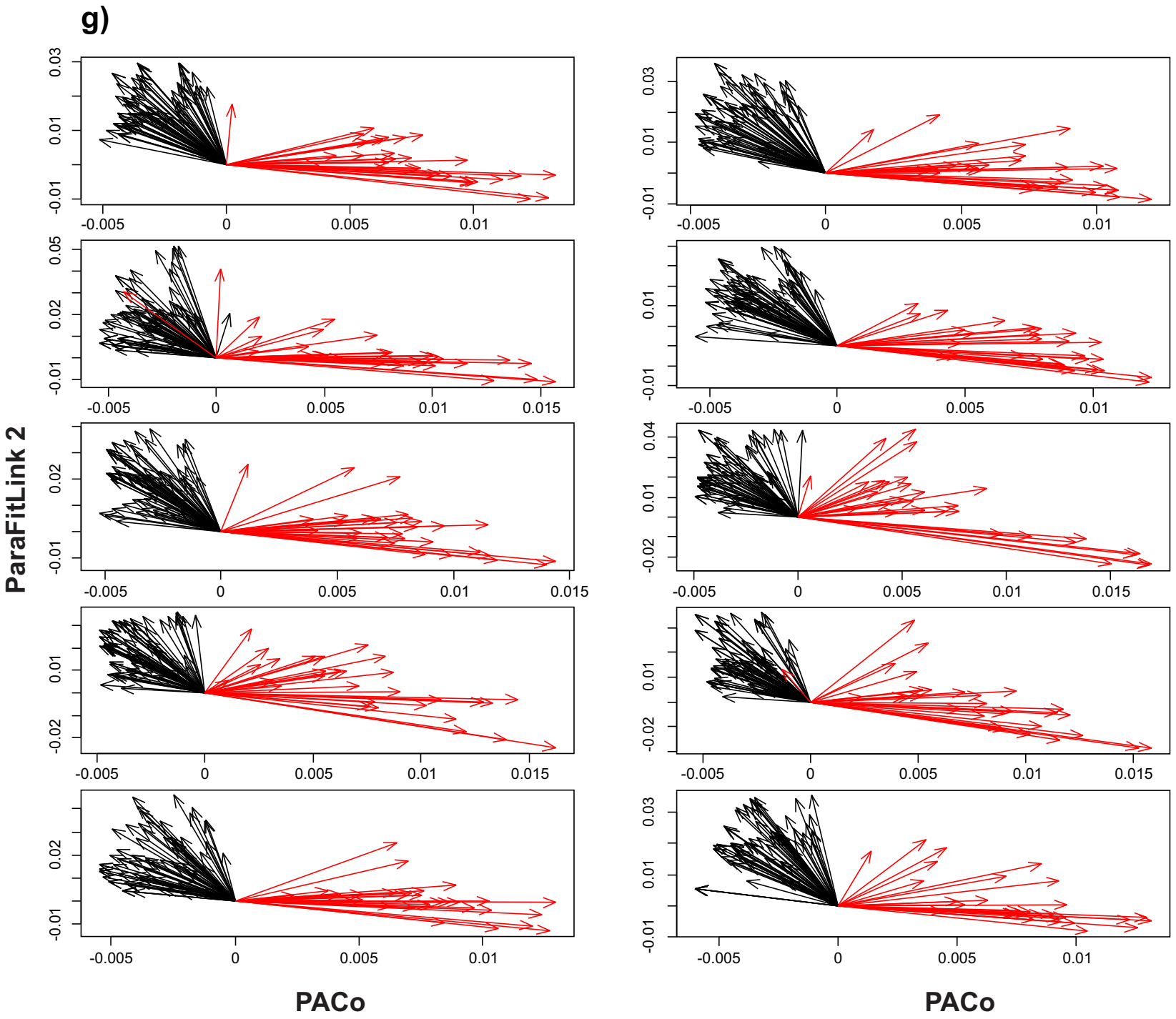
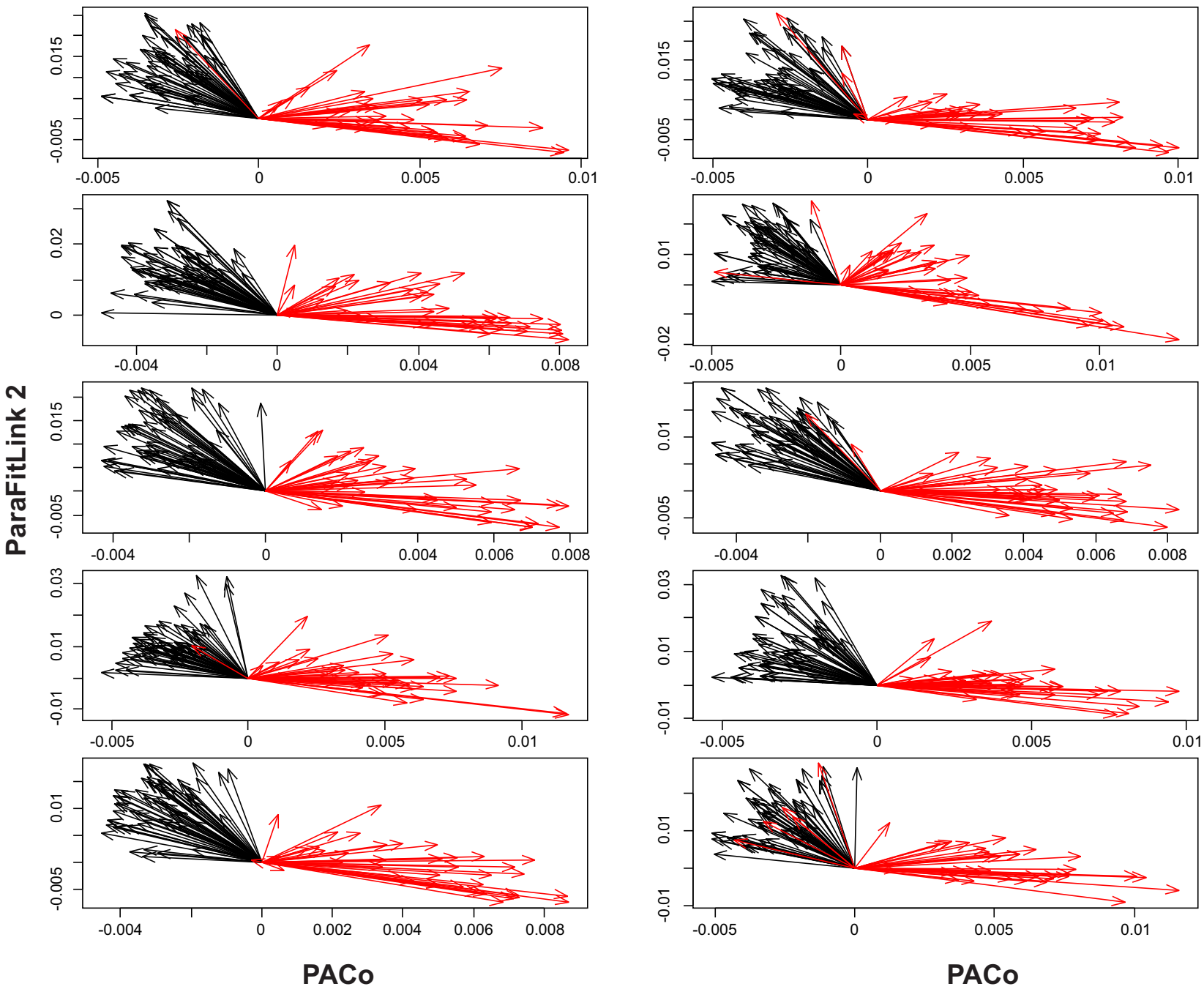
ParaFitLink 2
PACo
PACo

f)




g)
ParaFitLink 2
PACo
PACo

h)



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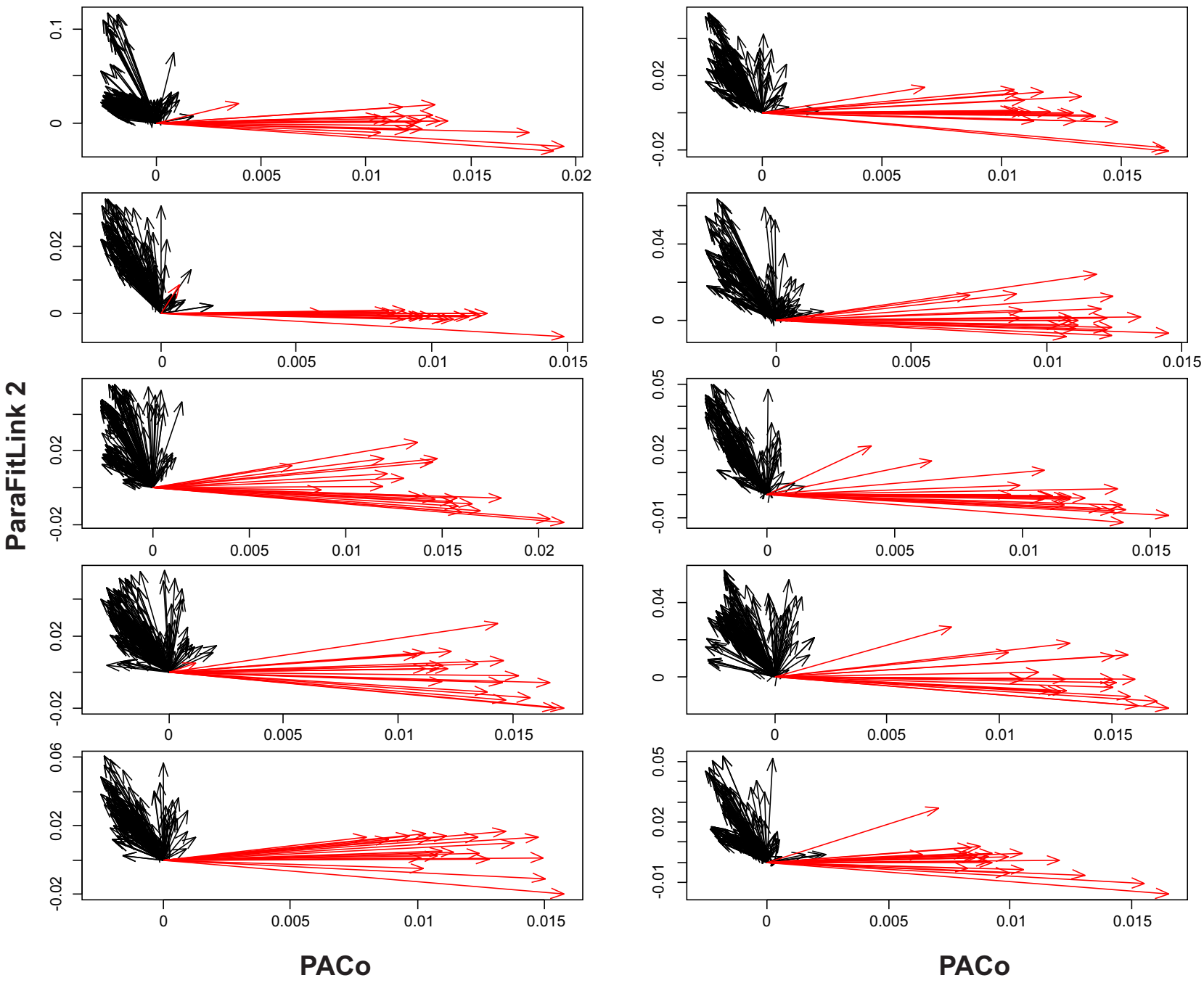

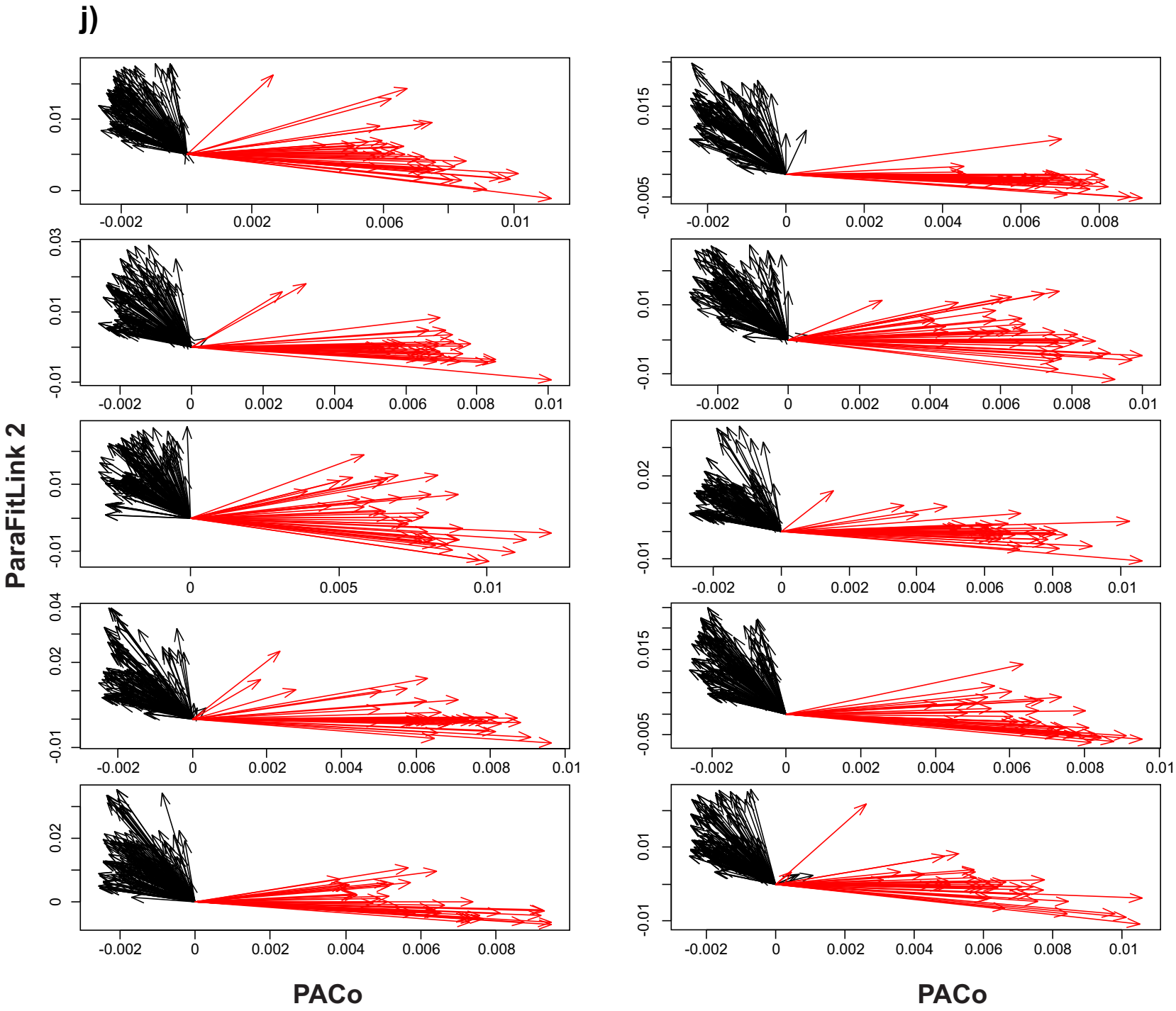
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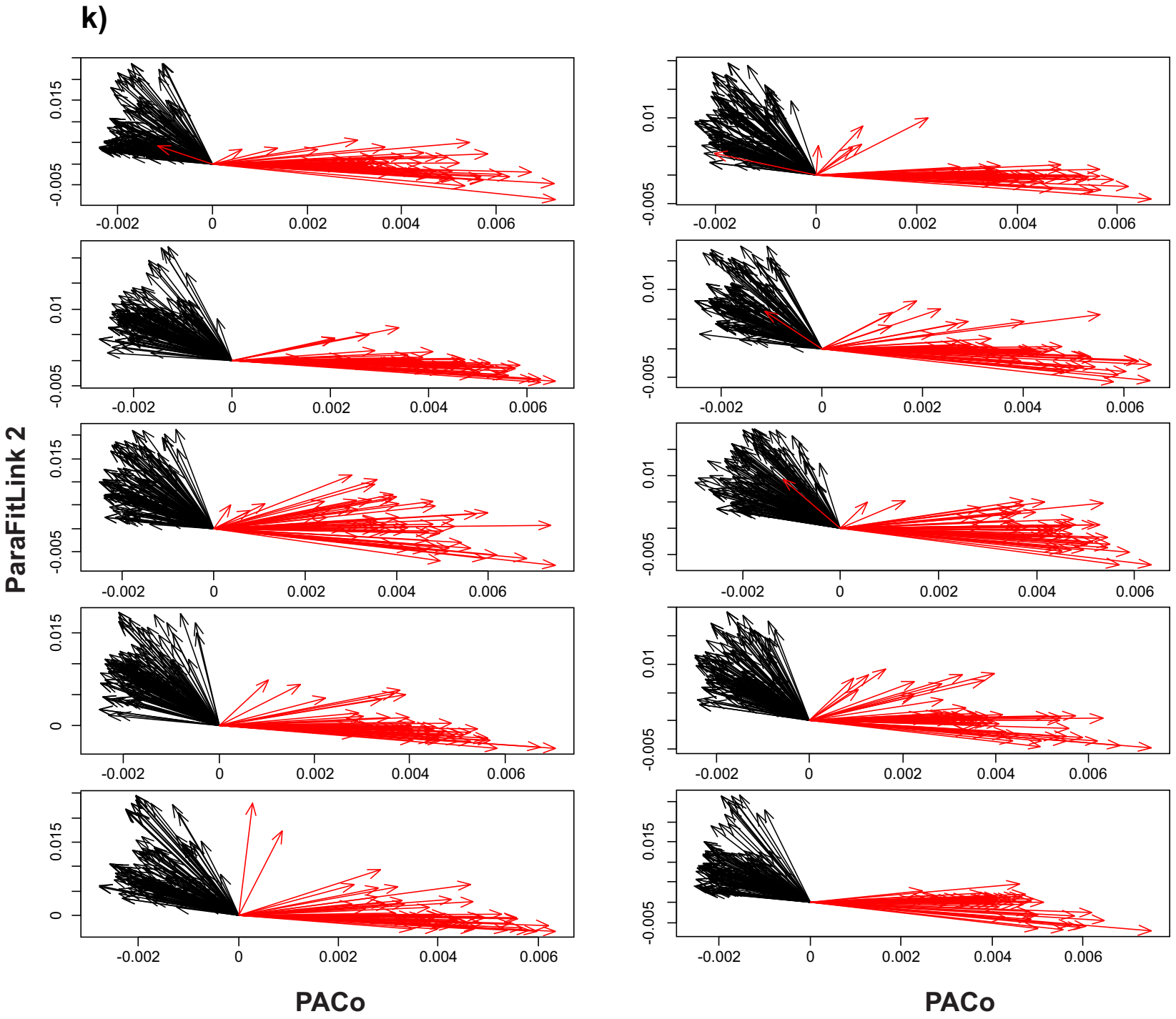
ParaFitLink 2
PACo
PACo


k)
ParaFitLink 2
PACo
PACo

I)

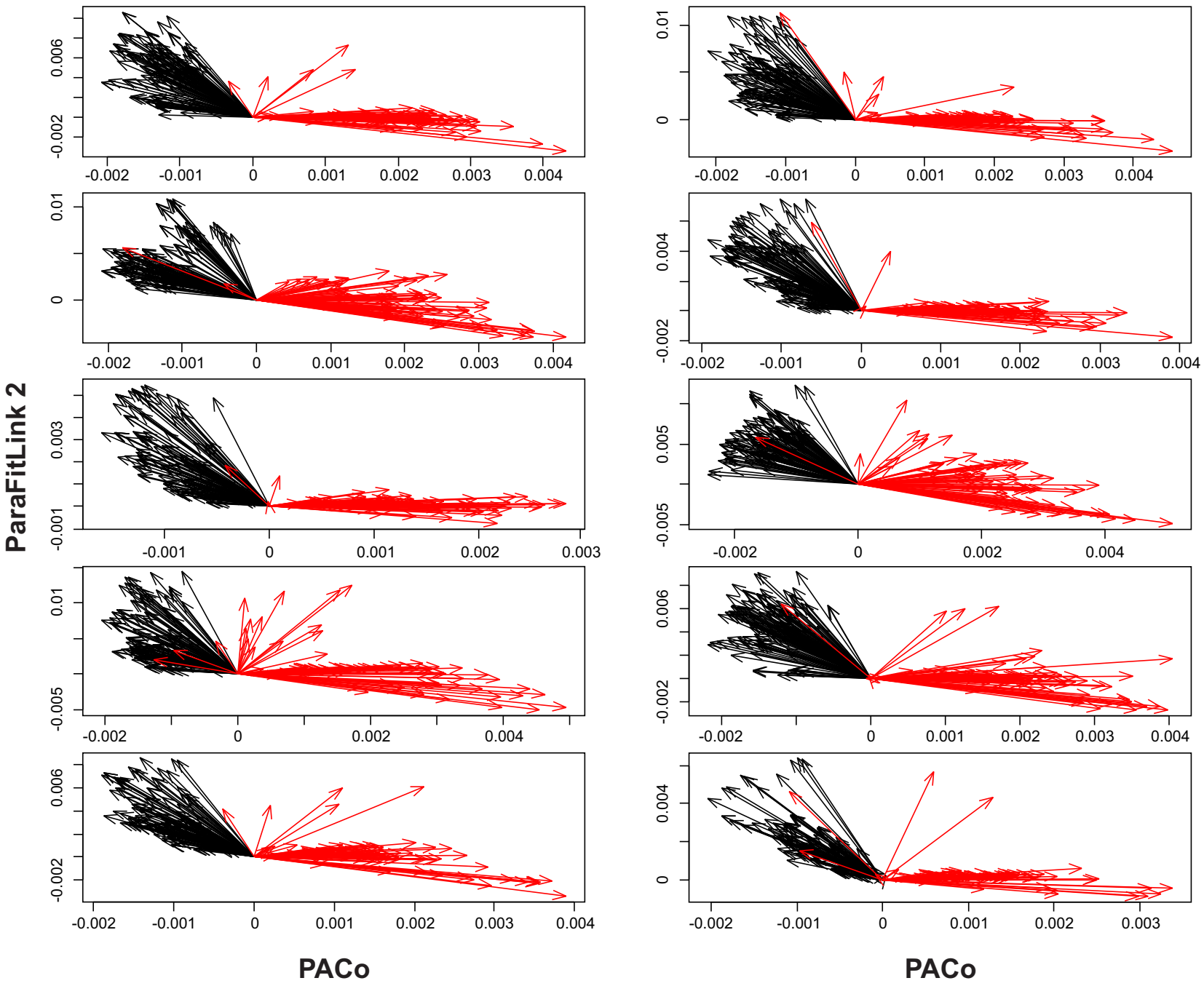


Figure S3. Normalized squared residual values ε_i^2) of individual 'o'-/'n'-associations obtained by PACo using additive trees. Pink bars indicate potential outlier associations identified by the pipeline. Taxa names in black, bold, and underlined represent OTUs retrieved by PACo that do not actually demonstrate phylogenetic distortion as in truly outlier associations.

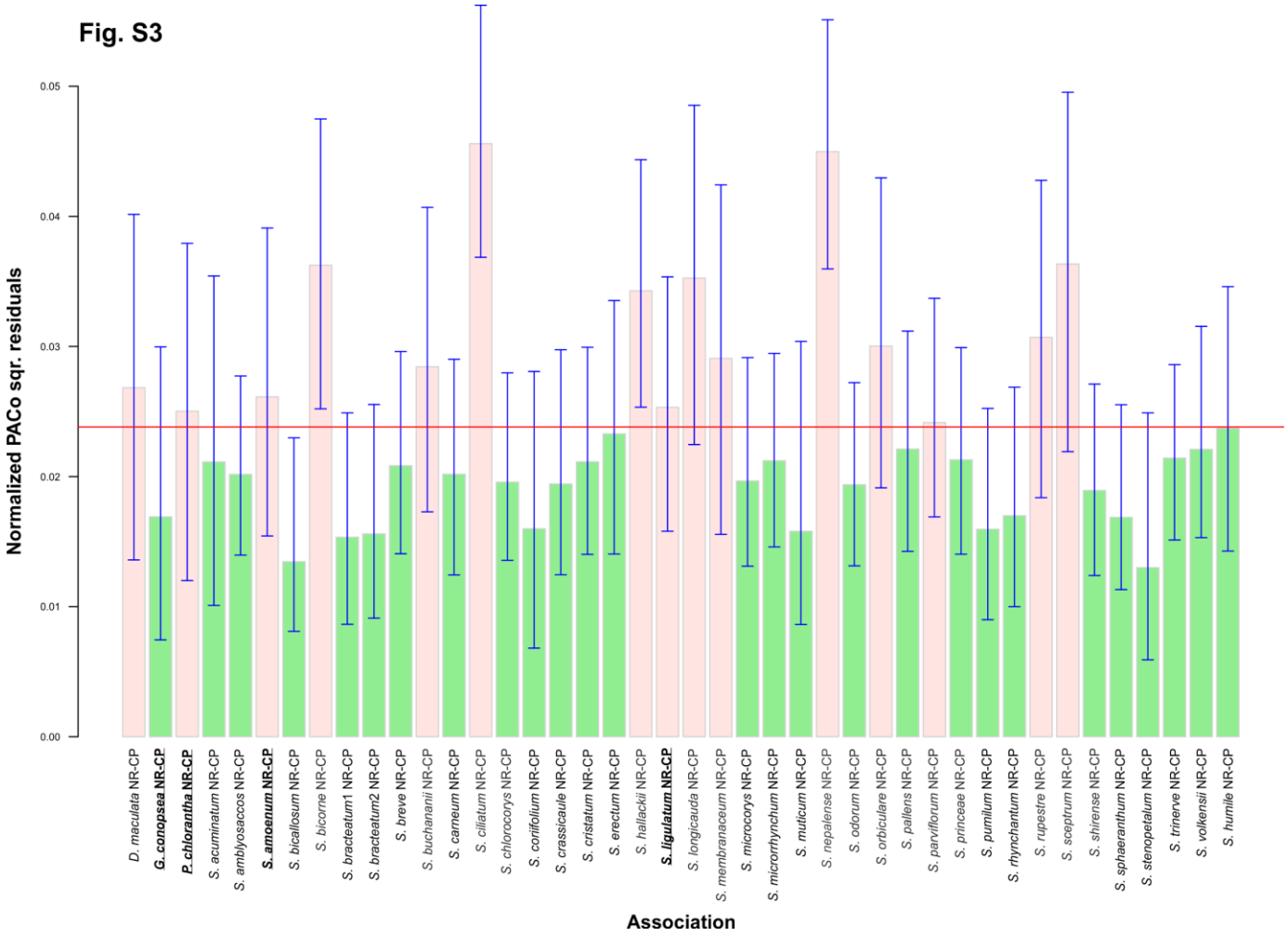


Figure S4. Cophylogenetic plot showing the nuclear (ITS, left) and chloroplast (*matK*, *trnL–trnF*, right) phylogenies of *Satyrium*. Bayesian posterior probabilities > 0.95 are shown above corresponding branches. Terminals in red, bold, and underlined represent associations identified by PACo as outliers that are indeed conflicting sequences. Terminals in black, bold, and underlined represent associations retrieved by PACo that do not actually demonstrate phylogenetic distortion as in truly conflicting associations

Fig. S4

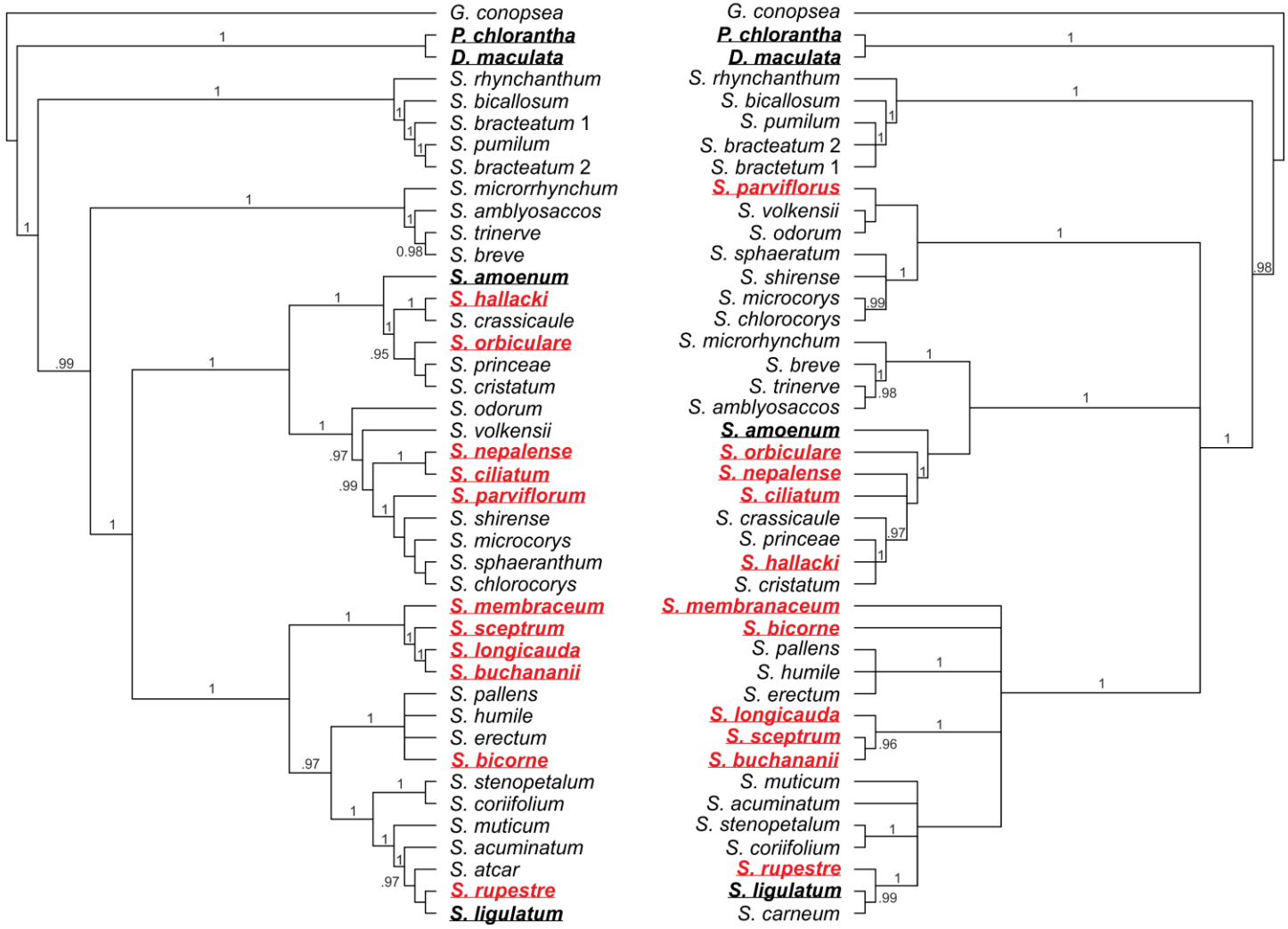


Figure S5. Cophylogenetic plot of nuclear (right) and chloroplast (left) trees of *Satyrium* showing outlier associations detected by PACo. Scale-color (bottom left) correspond to squared residual values ε_i^2 of individual 'o'-/'n'-associations. Potential outlier associations are indicated in purple, blue and light blue (see cutoff value 0.024 in Fig. S4).

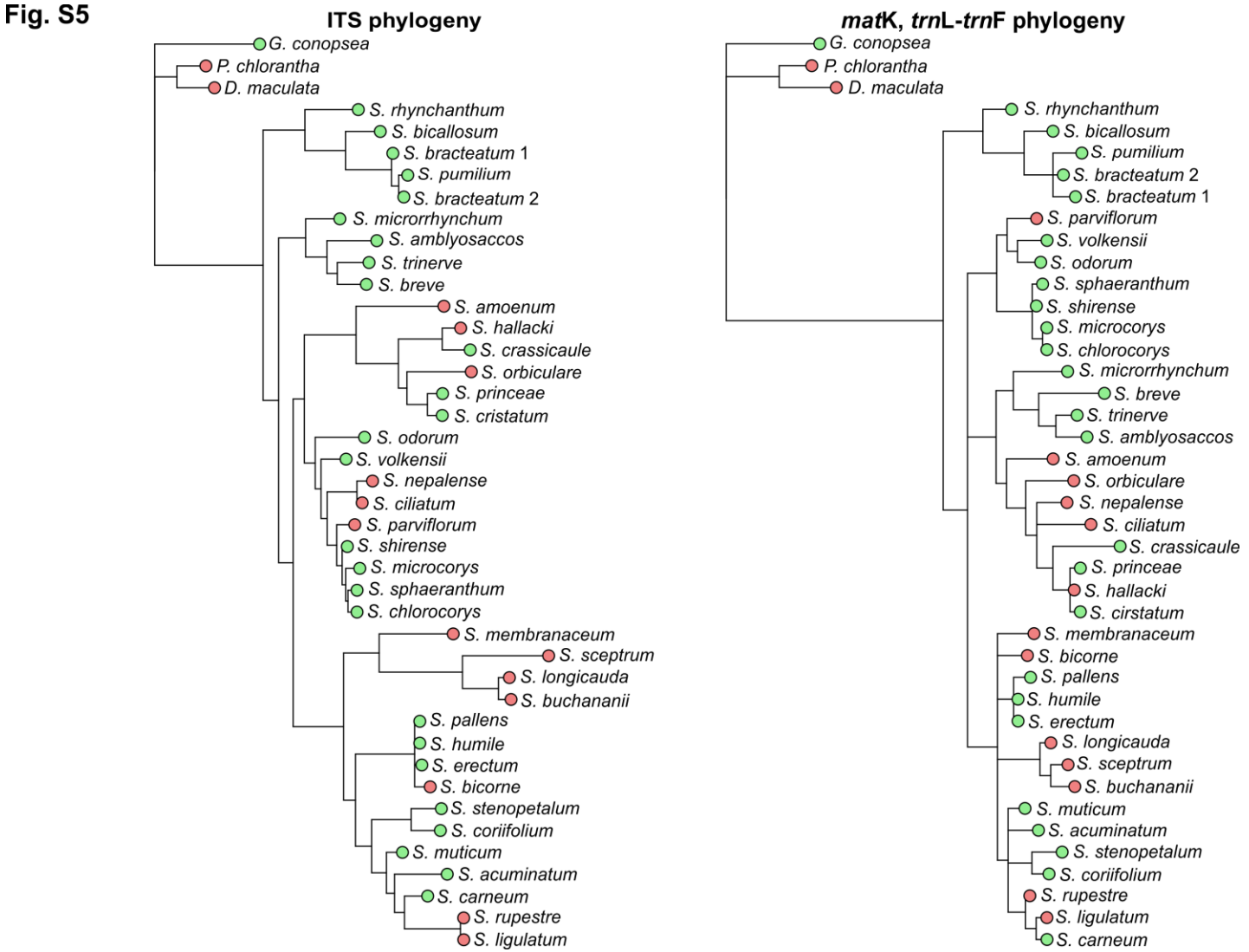


Figure S6. Normalized squared residual values ε_i^2 of individual associations obtained by PACo using simulated additive trees of 200 terminals, which 20% of those are conflicting. Pink bars indicate potential outlier associations identified by the pipeline, whereas light-green bars represent non-conflicting associations.

Fig. S6

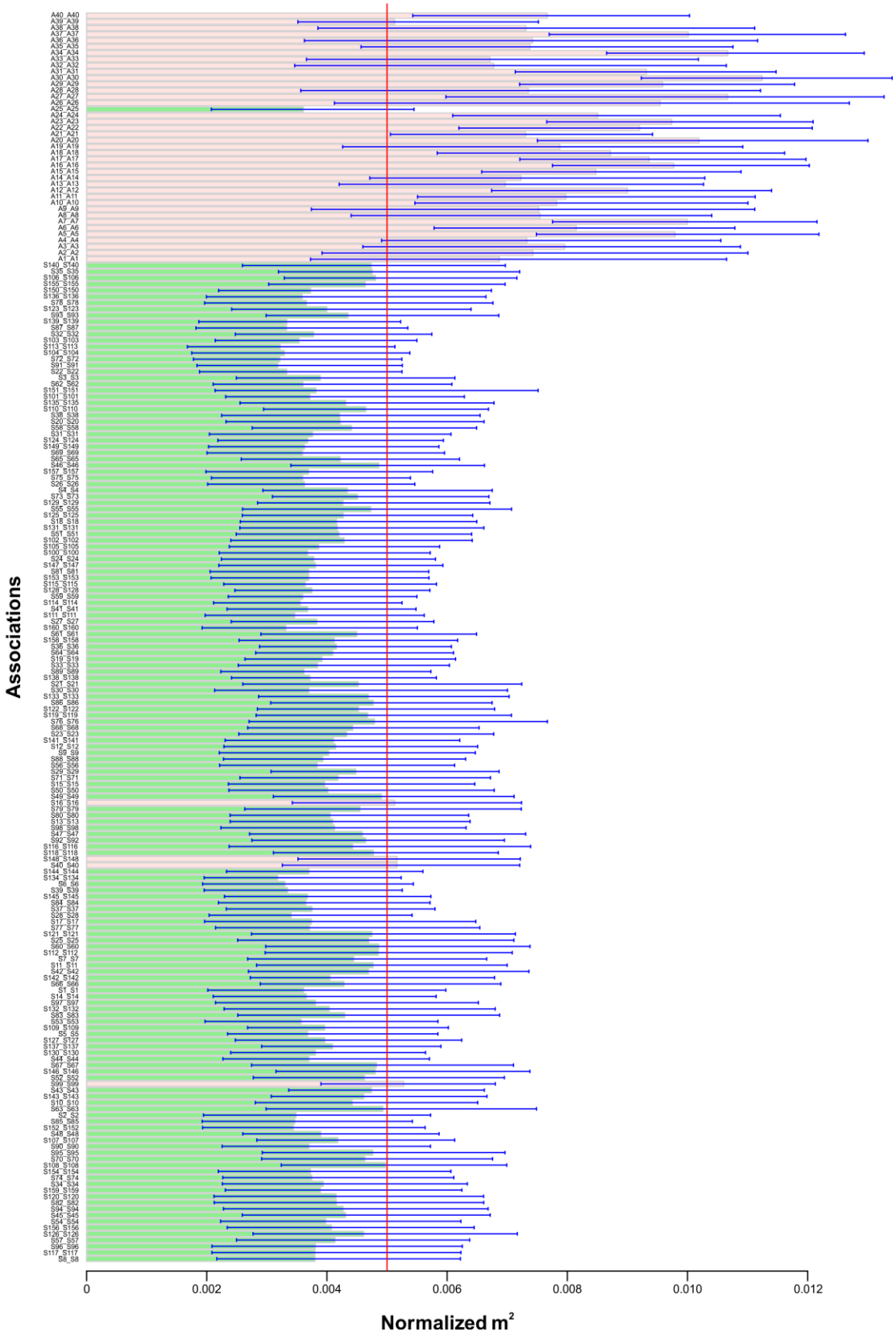


Figure S7. Cophylogenetic plot of two simulated gene trees showing outlier associations detected by PACo. Red circles on tips correspond to potential outliers, whose squared residual values ε_i^2 of individual associations are higher than the cutoff value (1/N). Non-conflicting associations are indicated in light-green circles.

Fig. S7

TABLES

Table S1. Primers and PCR settings used for amplifying chloroplast and nuclear DNA loci.

Loci	Primer	Sequence	Reference	Pre-melt	Amplification	Final extension	Number of amplification cycles
ITS	ITS 4	TCC-TCC-GCT-TAT-TGA-TAT-GC	Baldwin (1992)	95°C (3 min)	95°C (30 secs) + 52°C (1 min) + 68°C (1 min)	68°C (10 min)	39
	ITS 5	GGA-AGT-AAA-AGT-CGT-AAC-AAG-G		95°C (3 min)	95°C (30 secs) + 52°C (1 min) + 68°C (1 min)	68°C (10 min)	39
ETS	EST-Orchid	CAT-ATG-AGT-TGT-TGC-GGA-CC (AT)-T	Monteiro et al (2010)	95°C (3 min)	95°C (30 secs) + 52°C (1 min) + 68°C (1 min)	68°C (10 min)	39
	18-IGS	AGA-CAA-GCA-TAT-GAC-TAC-TGG-CAG-G	Markos and Balwin (1998)	95°C (3 min)	95°C (30 secs) + 52°C (1 min) + 68°C (1 min)	68°C (10 min)	39
Xdh	X502F	TGT-GAT-GTC-GAT-GTA-TGC	Górniak et al (2010)	95°C (3 min)	95°C (30 secs) + 53°C (1 min) + 68°C (1.5 min)	68°C (10 min)	39
	X1599R	G(AT)G-AGA-GAA-A(CT)TG-GAG-CAA-C		95°C (3 min)	95°C (30 secs) + 53°C (1 min) + 68°C (1.5 min)	68°C (10 min)	39
Ycf1	3720F	TAC-GTA-TGT-AAT-GAA-CGA-ATG-G	Neubig et al (2009)	95°C (3 min)	95°C (30 secs) + 54°C (1 min) + 68°C (1.5 min)	68°C (10 min)	39
	5500R	GCT-GTT-ATT-GGC-ATC-AAA-CCA-ATA-GCG		95°C (3 min)	95°C (30 secs) + 54°C (1 min) + 68°C (1.5 min)	68°C (10 min)	39
*trn*S-G	trn-S(GCU)	GCC-GCT-TTA-GTC-CAC-TCA-GC	Hamilton (1999)	95°C (3 min)	95°C (30 secs) + 51.5°C (1 min) + 68°C (1.5 min)	68°C (10 min)	39
	trn-G(UCC)	GAA-CGA-ATC-ACA-CTT-TTA-CCA-C		95°C (3 min)	95°C (30 secs) + 51.5°C (1 min) + 68°C (1.5 min)	68°C (10 min)	39

Table S2. Species names and voucher information for material used in this study. Taxa sequenced in this study are indicated in bold letters.

			Nuclear - ribosomal dataset			Chloroplast dataset		
Taxon	**DNA Source - voucher**	**Distribution**	**ITS spacer**	**ETS spacer**	***Xdh* gene**	***matK* gene**	***Trn*S-G spacer**	***ycf1* gene**
***Catasetum collare* Cogn.**	cult. BGM[1] 5/1000 (M)	Brasil, Colombia, Ecuador, Venezuela	KT768384	KT768350	KT768454	-	KT768421	KT768491
***Catasetum juruenense* Hoehne**	cult. BGM 5/1223 (M)	Brazil	KT768385	KT768351	KT768455	-	KT768422	KT768492
***Catasetum macrocarpum* Rich. ex Kunth**	cult. BGM 96/3071 (M)	Brazil-Venezuela	KT768386	KT768352	KT768456	-	KT768423	KT768493
***Catasetum meeae* Pabst**	cult. BGM 97/3836 (M)	Brazil	KT768387	KT768353	KT768457	-	-	-
***Catasetum* x *roseoalbum* (Hook.) Lindl.**	cult. BGM 6/2496 (M)	Venezuela	KT768388	KT768354	KT768458	-	KT768424	KT768494
Catasetum sp. 1	ML086	-	JF692010	-	-	-	-	JF692138
Catasetum sp. 2	ML301	-	JF692017	-	-	-	-	JF692140
Catasetum sp. 3	SR1153	-	JF691914	-	-	-	-	JF692061
Catasetum sp. 4	SR1203	-	JF691923	-	-	-	-	JF692066
Catasetum sp. 5	SR1213	-	JF691925	-	-	-	-	JF692067
Catasetum sp. 6	SR1463	-	JF691960	-	-	-	-	JF692150
***Clowesia russelliana* (Hook.) Dodson**	cult. BGM 98/2889 (M)	Central America, Colombia, Venezuela	KT768389	-	-	-	KT768425	KT768495
Clowesia sp. 1	SR0703	-	JF69204	-	-	-	-	JF692131
Clowesia sp. 2	SR0716	-	JF692041	-	-	-	-	JF692154
Clowesia sp. 3	SR0726	-	JF692042	-	-	-	-	JF692155

Cyanaeorchis arundinae (Rchb. f.) Barb. Rodr.	Klein 126	Brazil	KF771817	-	-	KF771821	-	-
Cyanaeorchis minor Schltr.	Klein 124	Brazil	KF771818	-	-	KF771822	-	-
Cyanaeorchis praetermisa J.A.N.Bat. & Bianch.	Batista et al. 3041 (BHCB)	Brazil	KF771819	-	-	KF771823	-	-
***Cycnoches aureum* Lindl. & Paxton**	Pérez & Gerlach 1473 (M)	Panama	KT768390	KT768355	KT768459	-	KT768426	KT768496
***Cycnoches barthiorum* G.F.Carr & Christenson**	cult. BGM 12/1476 (M)	Colombia	KT768391	KT768356	KT768460	-	KT768427	KT768497
***Cycnoches chlorochilon* Klotzch**	cult. BGM 94/981 (M)	Panama, Colombia, Venezuela	KT768392	KT768357	KT768461	-	KT768428	KT768498
***Cycnoches cooperi* Rolfe**	Whitten W3591 (FLAS)	Brazil, Peru	KT768393	KT768358	KT768462	-	KT768429	KT768499
***Cycnoches densiflorum* Rolfe**	cult. BGH[2] Kusibab 5/2004	Colombia, Panama	KT768394	KT768359	KT768463	-	KT768430	KT768500
***Cycnoches dianae* Rchb. f.**	Pérez & Gerlach 1468 (M)	Panama	KT768395	KT768360	KT768464	-	KT768431	KT768501
***Cycnoches egertonianum* Bateman**	(1) Franke s.n. (MEXU)	Southern Mexico, Guatemala, Belize, Honduras	KT768397	KT768362	KT768466	-	KT768433	KT768503
	(2) cult. BGM 12/1471 (M)	Southern Mexico, Guatemala, Belize, Honduras	KT768396	KT768361	KT768465	-	KT768432	KT768502

***Cycnoches guttulatum* Schltr.**	Pérez & Gerlach 1476 (M)	Panama	KT768398	KT768363	KT768467	-	KT768434	KT768504
***Cycnoches haagii* Barb. Rodr.**	cult. BGH Brock 10/72	Surinam, Venezuela, Colombia, Ecuador, Brazil, Peru, Bolivia	KT768399	KT768364	KT768468	-	KT768435	KT768505
***Cycnoches herrenhusanum* Jenny & G.A. Romero**	cult. BGH Hubein 1/78	Colombia	KT768400	KT768365	KT768469	-	KT768436	KT768506
***Cycnoches lehmannii* Rchb. f.**	cult. BGH Portilla T1/97	Ecuador, Peru	KT768401	KT768366	KT768470	-	KT768437	KT768507
***Cycnoches loddigesii* Lindl.**	cult. BGH H9/70	Colombia, Surinam, Venezuela	KT768402	KT768367	KT768471	-	KT768438	KT768508
***Cycnoches manoelae* V.P. Castro & Campacci**	cult. BGM 12/2255 (M)	Brazil	KT768403	KT768368	KT768472	-	KT768439	KT768509
***Cycnoches pachydactylon* Schltr.**	Pérez & Gerlach 1469 (M)	Panama	KT768404	KT768369	KT768473	-	KT768440	KT768510
***Cycnoches pentadactylon* Lindl.**	cult. BGH Kusibab 1/11	Brazil, Peru	-	KT768370	KT768474	-	KT768441	KT768511
***Cycnoches peruvianum* Rolfe**	(1) cult. BGM 12/0839 (M)	Ecuador, Peru, Colombia	KT768406	KT768372	KT768475	-	KT768443	KT768513
	(2) cult. BGH Kusibab 5/04	Ecuador, Peru, Colombia	KT768405	KT768371	-	-	KT768442	KT768512

***Cycnoches suarezii* Dodson**	cult. BGM 12/0836 (M)	Ecuador	KT768408	KT768374	KT768476	-	KT768444	KT768515
***Cycnoches ventricosum* Bateman**	cult. BGM 3/3992 (M)	Southern Mexico, Guatemala, Belize, Honduras, northern Nicaragua	KT768409	KT768375	KT768477	-	KT768445	KT768516
***Cycnoches warszewiczii* Rchb. f.**	cult. BGH H1/73	Southern Nicaragua, Costa Rica, Panama	KT768410	KT768376	KT768478	-	KT768446	KT768517
***Cycnoches* sp. 1**	Rodríguez s.n. (M)	-	KT768407	KT768373	-	-	-	KT768514
Cycnoches sp. 2	SR1106	-	JF691909	-	-	-	-	JF692056
Cycnoches sp. 3	SR1139a	-	JF691912	-	-	-	-	JF692059
Dressleria dilecta (Rchb.f.) Dodson	Whitten 1019 (FLAS)	Colombia, Panama, Costa Rica, Nicaragua	AF239411	-	-	AF239507	-	EU490731.1
***Dressleria* sp.**	cult. BGM 11/1194 (M)	-	KT768413	KT768377	-	-	-	KT768521
Galeandra devoniana R.H. Schomb. ex Lindl.	(1) Silva 1373 (HUEFS); (2) Pupulin 1133 (JBL)	Brazil, Colombia, Guyana, Venezuela	(1) EU877142	(2) EU877125	-	(2) KF660268	-	(2) KF660330
Galeandra sp.	ML092	-	JF692011	-	-	-	-	JF692079
Grobya galeata Lindl.	MWC295	Brazil	AF470487	-	-	AF47045	-	-
***Mormodes badia* Rolfe ex Watson**	cult. BGM 2/2480 (M)	Mexico	KT768415	KT768380	KT768484	-	KT768450	KT768525

***Mormodes ephippilabia* Fowlie**	cult. BGM 3/0775 (M)	Honduras	KT768416	KT768381	KT768485	-	-	KT768526
***Mormodes luxata* Lindl.**	cult. BGM 92/3103 (M)	Mexico	KT768417	KT768382	KT768486	-	-	KT768527
***Mormodes punctata* Rolfe**	Pérez & Gerlach 1483 (M)	Panama	KT768418	KT768383	KT768487	-	-	KT768528
Outgroup								
***Ansellia africana* Lindl.**	cult. BGM X/0021 (M)	Sub-saharan Africa	-	-	KT768453	-	KT768420	KT768490
***Cymbidium eburneum* Lindl.**	cult. BGM (M)	Burma, China, India, Nepal, Vietnam	KT768411	-	KT768479	-	KT768447	KT768518
***Cymbidium tracyanym* Rolfe**	cult. BGM (M)	Burma, China, Thailand, Vietnam	KT768412	-	KT768480	-	-	KT768519
Cyrtopodium andersonii (Lamb. ex Andrews) R. Br.	(1) Chase O-341; (2) Chase "no voucher" (K)	Brazil, Colombia, Guyana, Surinam, Venezuela	(1) AF470490	-	-	(1) AF470460	-	(2) KF660329
Cyrtopodium punctatum (L.) Lindl.	Chase O-126 (K)	Middle-north South America to Mexico	AF239412	-	-	AF239508	-	-
***Eulophia petersii* Rchb. f.**	cult. BGM 11/3892 (M)	South Africa	-	-	KT768481	-	KT768448	KT768522
***Grammatophyllum measuresianum* Sander**	cult. BGM Stoch 6/95 (M)	Philippines	-	KT768379	KT768483	-	KT768449	KT768524

***Oeceoclades maculata* (Lindl.) Lindl.**	cult. BGM 96/4473 (M)	Tropical America, Africa	-	-	KT768488	-	KT768451	KT768529
***Oeceoclades pulchra* (Thouars) M.A.Clem. & P.J. Cribb**	cult. BGM X/434 (M)	Tropical Asia, Asutralia	KT768414	-	KT768482	-	-	KT768523
***Oncidium luteum* Rolfe**	cult. BGM 13/0100 (M)	Costa Rica - Panama	KT768419	-	KT768489	-	KT768452	KT768530

Table S3. Results of jModel test.

Data partition	AIC	LRT
ITS	GTR+Γ	GTR+Γ
ETS	TPM2uf+Γ	GTR+Γ
Xdh	HKY+Γ	GTR+Γ
matK	TVM+Γ	GTR+Γ
trnS-trnG	TVM1+Γ	GTR+Γ
ycf1	TVM+Γ	GTR+Γ

Table S4. Number of misclassified congruent ('c') and outlier ('x') associations in 10 pairs of simulated additive and unit branch length gene trees based on the median values of PACo and ParaFitLink2 (PFL2) statistics using the Partionioning Around Medioids algorithm (PAM). Trees were simulated with a) 50, b) 100 and c) 200 and a corresponding number of 10%, 20%, 30% and 40% of outlier OTUs, respectively. For each pair of trees, PACo and ParaFit were applied to 1000 sets of post burn-in trees obtained from Bayesian inferences by computing median statistics. PAM was applied for separation between 'c' and 'o' links using PACo in combination with ParaFit, or only the PACo statistic. Values of the average silhouette width (S) for each tree are also reported, as well as the total number of misidentified associations (Mis.T) and Average Silhouette width value (Av.S). Boldfaced values correspond to cases where the PAM algorithm required k=3 to separate 'x' associations, given that PFL2 tended to separate 'c' associations into two artificial clusters.

Table S4

(A)

	Proportion of incongruent associations (%)																											
	10								20								30								40			
	Additive tree				Branch lengths = 1				Additive tree				Branch lengths = 1				Additive tree				Branch lengths = 1				Additive tree			
	PACo+PFL2		PACo		PACo+PFL2		PACo		PACo+PFL2		PACo		PACo+PFL2		PACo		PACo+PFL2		PACo		PACo+PFL2		PACo		PACo+PFL2		PACo	
Tree	**x**	**c**	**x**	**c**	**x**	**c**	**x**	**c**	**x**	**c**	**x**	**c**	**x**	**c**	**x**	**c**	**x**	**c**	**x**	**c**	**x**	**c**	**x**	**c**	**x**	**c**	**x**	**c**
1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0	0	0	1	0	1	0	2	0	2	2	3	0	2	2
S	**0.68**		0.91		0.68		0.87		0.73		0.87		**0.65**		0.83		0.66		0.84		0.6		0.81		0.56		0.67		0.6		0.66	
2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	2	0	1	0	4	0	4	0
S	0.75		0.91		0.73		0.89		0.66		0.8		0.62		0.8		0.65		0.77		0.62		0.77		0.6		0.69		0.55		0.72	
3	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	2	0	2	0	5	0	2	0	1	0	5	0	1	4	1	0
S	0.73		0.89		0.73		0.89		0.76		0.86		0.62		0.83		0.64		0.8		0.59		0.76		0.61		0.71		0.51		0.67	
4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	2	0	1	0	0	0	3	0	5	0	4	0
S	0.73		0.89		0.66		0.86		0.73		0.83		0.7		0.81		0.65		0.8		0.63		0.78		0.6		0.75		0.59		0.71	
5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	3	0	0	0	3	0	0	0	6	0	5	0
S	0.71		0.89		0.68		0.86		0.68		0.82		0.73		0.85		0.65		0.8		0.65		0.76		0.65		0.7		0.57		0.63	
6	1	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0	2	0	1	0	2	0	2	0	2	0	2	0	6	0	4	0
S	0.8		0.89		0.75		0.9		0.72		0.89		0.67		0.85		0.67		0.77		0.6		0.8		0.64		0.77		0.63		0.72	
7	0	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
S	0.75		0.93		**0.6**		0.88		0.72		0.87		0.67		0.79		0.67		0.82		0.61		0.8		0.56		0.72		0.53		0.73	
8	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	1	0	1	0	6	0	2	0	3	0	3	0	3	0	4	0
S	**0.74**		0.92		**0.65**		0.88		0.62		0.82		0.59		0.81		0.69		0.8		0.66		0.78		0.64		0.79		0.6		0.71	
9	0	0	0	0	1	0	0	0	0	0	0	0	3	0	0	0	0	0	1	0	1	0	1	0	0	0	2	1	6	0	1	1
S	0.71		0.87		0.74		0.86		0.67		0.84		0.62		0.8		0.56		0.74		0.53		0.77		0.54		0.7		0.48		0.61	
10	0	0	0	0	0	0	0	0	0	0	1	0	2	0	1	0	0	0	2	0	1	0	3	0	0	0	3	0	2	0	3	0
S	0.79		0.91		0.7		0.91		0.73		0.82		0.7		0.79		0.66		0.77		0.6		0.78		0.61		0.73		0.56		0.71	
Mis. T	1	0	0	0	2	0	1	0	1	0	1	0	9	0	3	0	8	0	8	0	22	0	13	0	13	0	21	3	36	4	28	3
Av. S	0.739		0.901		0.692		0.88		0.702		0.842		0.657		0.816		0.65		0.791		0.609		0.781		0.601		0.723		0.562		0.687	

(B)

	Proportion of incongruent associations (%)																													
	10								20								30								40					
	Additive tree				Branch lengths = 1				Additive tree				Branch lengths = 1				Additive tree				Branch lengths = 1				Additive tree				Branch lengths = 1			
	PACo+PFL2		PACo		PACo+PFL2		PACo		PACo+PFL2		PACo		PACo+PFL2		PACo		PACo+PFL2		PACo		PACo+PFL2		PACo		PACo+PFL2		PACo		PACo+PFL2		PACo	
Tree	**x**	**c**	**x**	**c**	**x**	**c**	**x**	**c**	**x**	**c**	**x**	**c**	**x**	**c**	**x**	**c**	**x**	**c**	**x**	**c**	**x**	**c**	**x**	**c**	**x**	**c**	**x**	**c**	**x**	**c**	**x**	**c**
1	0	0	0	0	0	0	0	0	1	0	1	0	2	0	2	0	1	0	0	0	1	0	1	0	1	0	2	0	3	0	3	0
S	0.73		0.92		0.74		0.93		0.69		0.83		0.68		0.86		0.72		0.84		0.72		0.84		0.62		0.72		0.61		0.78	
2	0	0	0	0	0	0	0	0	1	0	0	0	3	0	2	0	0	0	0	0	2	0	1	0	3	0	3	0	3	0	5	0
S	0.75		0.92		0.74		0.91		0.63		0.82		0.63		0.86		0.66		0.8		0.68		0.83		0.66		0.76		0.61		0.75	
3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	2	0	5	0	4	0	3	0	2	0	6	0	3	0
S	0.7		0.9		**0.58**		0.8		0.7		0.87		0.71		0.88		0.68		0.89		0.6		0.79		0.63		0.76		0.61		0.77	
4	0	0	0	0	1	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	3	0	0	0	1	0	1	0	11	0	3	0
S	**0.63**		0.91		0.75		0.92		0.66		0.8		0.67		0.85		0.7		0.85		0.7		0.83		0.61		0.72		0.55		0.74	
5	0	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0	1	0	0	0	2	0	1	0	2	0	1	2	2	0	0	0
S	0.72		0.9		0.7		0.9		0.68		0.85		0.66		0.86		0.69		0.83		0.67		0.84		0.63		0.75		0.59		0.76	
6	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	3	0	1	0	4	0	1	0	1	0	3	0	8	2	6	5
S	0.7		0.89		0.7		0.89		0.71		0.83		0.69		0.86		0.66		0.81		0.57		0.77		0.71		0.78		0.51		0.6	
7	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	2	0	1	0	2	0	1	0	5	0	2	0
S	0.74		0.94		0.7		0.92		0.76		0.88		0.72		0.88		0.68		0.81		0.58		0.77		0.67		0.76		0.61		0.77	
8	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	4	0	1	0	3	0	1	0	2	0	3	0	2	0	0	0
S	0.76		0.91		0.75		0.91		0.71		0.82		0.7		0.85		0.68		0.81		0.65		0.88		0.69		0.78		0.6		0.79	
9	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0	0	4	0	2	0	4	0
S	0.71		0.88		0.74		0.91		0.69		0.87		0.67		0.88		0.65		0.8		0.62		0.82		0.65		0.76		0.64		0.77	
10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4	0	1	0	5	0	7	0	6	0	5	0
S	0.7		0.9		0.74		0.91		0.69		0.86		0.69		0.87		0.7		0.84		0.68		0.81		0.66		0.76		0.64		0.77	
Mis. T	0	0	0	0	2	0	1	0	3	0	1	0	9	0	7	0	12	0	4	0	27	0	11	0	20	0	27	2	48	2	31	5
Av. S	0.714		0.907		0.714		0.9		0.692		0.843		0.682		0.865		0.682		0.828		0.647		0.818		0.653		0.755		0.597		0.75	

(C)

	Proportion of outliers (%)																											
	10								20								30								40			
	Additive tree				Branch lengths = 1				Additive tree				Branch lengths = 1				Additive tree				Branch lengths = 1				Additive tree			
	PACo+PFL2		PACo		PACo+PFL2		PACo		PACo+PFL2		PACo		PACo+PFL2		PACo		PACo+PFL2		PACo		PACo+PFL2		PACo		PACo+PFL2		PACo	
Tree	**x**	**c**	**x**	**c**	**x**	**c**	**x**	**c**	**x**	**c**	**x**	**c**	**x**	**c**	**x**	**c**	**x**	**c**	**x**	**c**	**x**	**c**	**x**	**c**	**x**	**c**	**x**	**c**
1	0	0	0	0	1	0	1	0	1	0	0	0	1	0	0	0	0	0	0	0	2	0	2	0	2	0	4	0	4	0	4	0
S	0.69		0.89		0.73		0.91		0.7		0.88		0.69		0.88		0.66		0.82		0.64		0.86		0.65		0.8		0.68		0.82	
2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3	0	1	0	7	0	6	0	3	0	4	0	4	0	2	0
S	0.72		0.92		0.72		0.92		0.73		0.88		0.73		0.91		0.66		0.81		0.7		0.87		0.62		0.8		0.64		0.81	
3	0	0	0	0	1	0	1	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	2	0	1	0	1	0
S	0.74		0.93		0.74		0.91		0.71		0.88		0.68		0.88		0.7		0.81		0.73		0.84		0.66		0.81		0.63		0.8	
4	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	3	0	1	0	2	19	8	1	24	0	2	0
S	0.72		0.91		0.69		0.9		0.69		0.87		0.7		0.88		0.68		0.82		0.67		0.85		0.59		0.62		0.58		0.78	
5	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0	0	0	3	0	2	0	33	1	22	5	2	0	3	0
S	0.72		0.92		0.54		0.92		**0.72**		0.89		0.68		0.88		0.65		0.82		0.61		0.86		0.5		0.63		0.57		0.76	
6	0	0	0	0	1	0	1	0	0	0	0	0	1	0	1	0	1	0	1	1	3	0	2	0	5	0	3	4	7	0	2	0
S	0.73		0.93		0.71		0.92		0.68		0.87		0.7		0.89		0.66		0.82		0.67		0.87		0.63		0.7		0.67		0.82	
7	0	0	0	0	1	0	1	0	1	0	0	0	0	0	0	0	1	0	0	0	2	0	1	0	15	5	9	5	13	0	8	0
S	0.71		0.91		0.73		0.91		0.68		0.87		0.68		0.88		0.76		0.84		0.73		0.87		0.5		0.65		0.57		0.77	
8	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	4	0	1	0	3	4	3	4	4	0	4	0
S	0.71		0.9		0.75		0.91		0.7		0.86		0.69		0.89		0.66		0.81		0.66		0.84		0.63		0.7		0.69		0.82	
9	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	2	0	0	0	2	0	2	0	2	0	5	0	4	0	1	0
S	0.71		0.91		0.72		0.92		0.7		0.84		0.75		0.9		0.67		0.81		0.67		0.86		0.58		0.76		0.6		0.76	
10	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0	4	0	8	0	4	11	3	0
S	0.73		0.92		0.5		0.89		**0.67**		0.84		0.69		0.89		0.67		0.81		0.69		0.88		0.59		0.76		0.54		0.72	
Mis. T	0	0	0	0	5	0	5	0	4	0	0	0	6	0	4	0	7	0	2	1	26	0	17	0	70	29	68	19	67	11	30	0
Av. S	0.718		0.914		0.683		0.911		0.698		0.868		0.699		0.888		0.677		0.817		0.677		0.86		0.595		0.723		0.617		0.786	

Table S5. Alignment characterization.

Loci	Length (bp)	Parsimony Informative Sites	Number of cells
ETS	475	149 / 32%	35/61
ITS	705	320 / 46%	57/61
Xdh	991	115 / 12%	37/61
matK	1721	76 / 4%	8/61
*trn*S-G	936	107 / 11%	34/61
ycf1	1643	209 / 8%	55/61

Chapter 6

SEX AND THE CATASETINAE (DARWIN'S FAVOURITE ORCHIDS)

Pérez-Escobar, O., Gottschling, M., Whitten, M.W., Salazar, G. and G. Gerlach

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Sex and the Catasetinae (Darwin's favourite orchids) ☆

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ABSTRACT

Two sexual systems are predominant in Catasetinae (Orchidaceae), namely protandry (which has evolved in other orchid lineages as well) and environmental sex determination (ESD) being a unique trait among Orchidaceae. Yet, the lack of a robust phylogenetic framework for Catasetinae has hampered deeper insights in origin and evolution of sexual systems. To investigate the origins of protandry and ESD in Catasetinae, we sequenced nuclear and chloroplast loci from 77 species, providing the most extensive data matrix of Catasetinae available so far with all major lineages represented. We used Maximum Parsimony, Maximum Likelihood and Bayesian methods to infer phylogenetic relationships and evolution of sexual systems. Irrespectively of the methods used, Catasetinae were monophyletic in molecular phylogenies, with all established generic lineages and their relationships resolved and highly supported. According to comparative reconstruction approaches, the last common ancestor of Catasetinae was inferred as having bisexual flowers (i.e., lacking protandry and ESD as well), and protandry originated once in core Catasetinae (comprising *Catasetum*, *Clowesia*, *Cycnoches*, *Dressleria* and *Mormodes*). In addition, three independent gains of ESD are reliably inferred, linked to corresponding loss of protandry within core Catasetinae. Thus, prior gain of protandry appears as the necessary prerequisite for gain of ESD in orchids. Our results contribute to a comprehensive evolutionary scenario for sexual systems in Catasetinae and more generally in orchids as well.

1. Introduction

Sexual systems in angiosperms display great diversity (Barret, 2013) that has attracted generations of naturalists, field botanists and, more recently, population geneticists and ecologists for almost two centuries (Devos et al., 2011). The term refers to "distribution and function of gamete-producing morphological structures" (Renner et al., 2007). Different selective pressures favouring, for example, outcrossing and therefore "optimal amount of recombination" (Bawa and Beach, 1981), and better resource re-allocation to male-/female reproductive functions (e.g., Charnov and Bull, 1977; Charnov, 1979), have been proposed to explain the great diversity of sexual systems.

As temporal differentiation of the two sexes, protandry is a widespread sexual system in angiosperms (De Jong et al., 2011; Renner, 2014). It is defined as a form of dichogamy, with earlier maturation of the staminate function in unisexual and bisexual flowers (Bertin and Newman, 1993; Forrest, 2014; Webb and Lloyd, 1986). Several hypotheses have been put forth regarding the evolutionary advantages of protandry, including avoidance of mutual interference between the staminate and pistillate structures and reduction of self-pollination rates among flowers of the same inflorescence (geitonogamy) (Webb and Lloyd, 1986; Bertin and Newman, 1993; Jersáková and Johnson, 2007). Despite the relative abundance of protandry in angiosperms (Bawa and Beach, 1981), its multiple evolutionary origins in time and space are still unclear. This might also refer to the absence of densely sampled, well resolved phylogenies and *in-situ* observations on such sexual system (Renner, 2014).

Environmental sex determination (ESD) is an extreme form of labile sex expression (also known as ontogenic sex change, plasticity or disphasy) (Renner, 2014) and describes sex change in a structurally bisexual but functionally unisexual system (i.e., angiosperm flower) in response to environmental constrains during an individual's life history (Schlessman, 1988; Korpelainen, 1998). Thus, ESD plants are able to produce staminate, pistillate or even bisexual

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flowers, either on the same or in separate individuals under certain environmental conditions. In animals, ESD has evolved in a wide range of lineages, including turtles and crocodiles (Janzen and Paukstis, 1991) and fish (e.g., Atherinidae: Conover and Kynard, 1981). In these lineages, temperature is a key factor regulating sex expression, although for specific clades some other variables such as pH and water quality might play an important role in sex determination (Korpelainen, 1990). Unlike protandry, plasticity in sex expression is remarkably rare, occurring in only ca. 250 species of angiosperms (Renner, 2014). When individual fitness (either male or female function) is strongly influenced by environmental factors, then ESD is favoured by natural selection (Charnov and Bull, 1977), because sessile organisms such as plants have no chance to change after establishment at a particular locality and habitat. Pioneering studies on sexual labile expression evolution (e.g., Renner and Won, 2001; Renner et al., 2007; see also Renner, 2014 for a review on sexual systems in angiosperms) in plant lineages such as *Acer* L. indicate that ESD might be inheritable, as it occurs in sister species. However, whether ESD evolutionary is a derived or rather the ancestral character state in orchids remains to be assessed.

Orchid pollination syndromes have received much attention of researchers for centuries (Tremblay et al., 2005), but little is still known about evolution of their sexual systems. Monoecy is a prevailing mechanism in orchids, yet the number of gains and losses of other sexual systems such as protandry and ESD is uncertain. Charles Darwin, who extensively documented and studied sexual systems in several plant species (Darwin, 1877), had a strong personal affinity to orchids, resulting in the publication of his seminal work on orchid pollination mechanisms (Darwin, 1877). He was thus pioneering on knowledge about sexual systems of *Catasetum* Rich. ex Kunth and *Cycnoches* Lindl., members of Catasetinae, which in Darwin's own words are "the most remarkable of all orchids" (Darwin, 1877: 211).

Catasetinae comprise approximately 290 species that are classified in eight generic lineages, namely *Catasetum*, *Clowesia* Lindl., *Cyanaeorchis* Barb.Rodr., *Cycnoches*, *Dressleria* Dodson, *Galeandra* Lindl., *Grobya* Lindl. and *Mormodes* Lindl. (Chase et al., 2015; Pérez-Escobar et al., 2015). They are distributed from southern Florida to southern Brazil, northern Argentina and the Antilles (Batista et al., 2014; Romero and Pridgeon, 2009). The remarkable diversity with respect to reproductive biology makes Catasetinae an excellent group to study evolution of sexual systems (including ESD and protandry) and pollination syndromes. In Catasetinae, protandry refers to the production of flowers, in which the pollinarium must be removed before pollinia can be deposited in the stigmatic cavity (Romero, 1990), and is present in all members of *Clowesia*, *Dressleria* and some species of *Mormodes* (Fig. 1). Environmental sex determination is an exceedingly rare system in orchids (and angiosperms) and occurs in *Catasetum*, *Cycnoches* and in the complementary species of *Mormodes* only. Most inflorescences of such species consist of functionally either male or female flowers, although they are also able to produce inflorescences with intermixed staminate and pistillate flowers (intermediate, non-functional bisexual flowers may occur rarely) (Fig. 1).

Sex expression in Catasetinae is entirely determined by environmental variables such as light intensity and substrate type (Gregg, 1983; Zimmerman, 2011). Unlike in animal lineages, sun light is the most important factor determining sex in flowers (Gregg, 1982). It stimulates ethylene production in reproductive structures, being as much as 100 times higher in inflorescences grown under direct sunlight than those grown under shade (Gregg, 1983). Ethylene is known to be a natural regulator of sex expression (Abeles et al., 1992) in several Cucurbitaceae species (e.g., *Cucumis sativus* L.: Malepszy and Niemirowicz-Szczytt, 1991; Rudich et al., 1972; *Cucurbita texana* (Scheele) A. Garay: Krupnick et al., 2000) and therefore, it might play the same regulatory role in orchids as well.

Phylogenetic trees are basic tools to shed light on the origin and evolution of specialised sexual systems in plants lineages such as Catasetinae. Analyses based on molecular and morphological data have repeatedly sustained the monophyly of Catasetinae (Batista et al., 2014; Freudenstein et al., 2004; Pérez-Escobar et al., 2015; Romero, 1990; Whitten et al., 2014), but their internal phylogenetic relationships have not been reliably resolved. However, three lineages are readily distinguished, namely *Grobya*, [*Cyanaeorchis*–*Galeandra*] and the remainder (or core) Catasetinae (Batista et al., 2014; Whitten et al., 2014; Pérez-Escobar et al., 2015). Notably, specialised sexual systems (i.e., protandry and ESD) occur in core Catasetinae only, as the remainder genera exhibit bisexual, adichogamous flowers. As the phylogenetic backbone is not resolved (see Whitten et al., 2014), it is unclear at present whether the last common ancestor (LCA) of core Catasetinae has exhibited unisexual flowers and ESD, or bisexual, adichogamous flowers. Based on a cladogram inferred from vegetative and reproductive morphological traits, Romero (1990) proposed that protandry and unisexuality (i.e., ESD) are equally likely as the condition of core Catasetinae's LCA. However, he favoured a scenario, in which protandry has originated once and ESD has evolved two times independently, once in *Catasetum* and again in *Cycnoches*–*Mormodes*'s LCA (Romero, 1990).

The lack of knowledge about sexual system evolution in orchids is primarily due to limited taxon sample and amount of sequence data. In this study, we use comparative phylogenetic and ancestral state reconstruction approaches to estimate the phylogenetic relationships of Catasetinae analysing sequence data from three nuclear ('n') and two chloroplast ('cp') loci of 77 out of ~290 extant species of Catasetinae. Using a solid, explicitly phylogenetic framework, we revisit the ideas of Romero (1990) that protandry has a single origin in Catasetinae, while ESD may have evolved independently multiple times. Our data matrix includes species exhibiting bisexual flowers, protandry and ESD, all of which are present in Catasetinae. We aim at the development of an evolutionary scenario of sexual systems in Darwin's favourite orchid lineage.

2. Materials and methods

2.1. Taxon sampling, DNA sequencing and phylogenetic analysis

Table S1 provides full species names, geographic origins, voucher specimens and GenBank accession numbers of sequences included in phylogenetic analyses. Genomic DNA was extracted from herbarium and fresh leaf material with the NucleoSpin® plant kit (Macherey–Nagel; Düren, Germany), following the manufacturer's protocol. We amplified and sequenced 'n' ribosomal external and internal transcribed spacers (ETS and ITS, respectively), a fragment of the 'n' gene *Xdh*, a ~1500 bp fragment of the 'cp' gene *ycf*1, as well as the 'cp' *trn*S–*trn*G intergenic spacer. Amplification settings and sequencing primers are specified in Table S2. PCR products were purified with the ExoSap clean-up kit (Fermentas; St. Leon-Rot, Germany), and sequencing reactions were run on an ABI 3130 capillary sequencer (Thermo Fisher Scientific; Waltham, USA) following the manufacturer's protocol. Sequence editing was carried out using Geneious software v. 7.1.7 (BiomattersCorporation; Auckland, New Zealand).

Each locus was aligned separately using MAFFT version 7.1 (Katoh and Standley, 2013). For aligning 'n' ribosomal DNA loci and 'cp' *trn*S–*trn*G spacer, secondary structure of molecules was taken into account (i.e., using the -qINSi option). Congruence between 'n' and 'cp' data sets was assessed following Pérez-Escobar et al. (2015), using the PACo application (Balbuena et al.,

Fig. 1. Diversity of sexual systems in Catasetinae. (A) Inflorescence of *Mormodes lineata* Bateman ex Lindl. with sexually dimorphic, functionally pistillate (below) and staminate (above) flowers. (B) Functionally staminate (right) and pistillate (left) flowers of *M. lineata*. (C) Sexually dimorphic, functionally staminate and (D) pistillate flowers of *Cycnoches guttulatum* Schltr. (E) Protandrous flower of *Mormodes maculata* (Klotzsch) L.O. Williams in staminate (left) and pistillate (right) phase. Photos: G. Salazar & O. Pérez.

2013). That procedure is now available as a pipeline (http://datadryad.org/review?doi=doi:10.5061/dryad.q6s1f) and it was also employed to identify and remove sequences from the 'cp' data set that were found to be conflicting with the 'n' data sets. Conflicting chloroplast sequences were removed from the concatenated alignment because often phylogenies derived from chloroplast sequence data are in conflict with evolutionary interpretations of morphology, which are in agreement with phylogenies inferred from nuclear loci (e.g. Nauheimer et al., 2012). In addition, sexuality is linked to eukaryotic cells but neither from chloroplast nor their bacterial ancestor (Lodé, 2012). After removing conflicting sequences, matrices of each locus were re-aligned, concatenated and analysed under three different phylogenetic methods (see below).

Analyses of the separate and concatenated datasets were carried out under Maximum Likelihood (ML), Maximum Parsimony (MP) and Bayesian criteria. The best-fitting evolutionary models for ML and Bayesian analyses (for each data partition) were selected from 44 models implemented in jModelTest version 2.1.6 (Darriba et al., 2012), employing the Likelihood Ratio Test (LRT) and the Akaike information criterion (AIC) (Table S3). The ML and Bayesian analyses were conducted with RAxML-HPC Blackbox version 8.0.0 (Stamatakis, 2014) and MrBayes version 3.2.2 (Ronquist et al., 2012), respectively, both run at the CIPRES Science Gateway computing facility (Miller et al., 2010). Bayesian inference was carried out with two independent runs of four Markov chain Monte Carlo (MCMC) analyses with 20 million generation each, sampling trees every 1000th generation, and using default prior settings. The performance and convergence of the Bayesian chains were verified using TRACER version 1.5 (Rambaut and Drummond, 2007). Ratchet parsimony analyses were implemented in Winclada version 1.0 (Nixon, 2002) using the following settings: heuristic search, uninformative characters deactivated, 500 iterations, holding 1 tree per iteration, amb-poly = default. Statistical support values (BPP: Bayesian posterior probabilities, LBS: ML bootstrap support, PBS: Parsimony Bootstrap Support) were drawn on the resulting majority-rule Bayesian consensus trees.

2.2. Ancestral state reconstructions and evolutionary pathway of sexual systems

Twenty-five of the 164 known species with ESD (15%) and 23 of 89 known protandrous species (25%) of core Catasetinae were included in the analyses. The sister group of the Catasetinae is unclear (Whitten et al., 2014), but we selected two representatives each of Eulophiinae and Cymbidiinae (both Cymbidieae) as outgroups, plus *Polystachya* Hook. (Vandae, Polystachyinae). In such lineages, both protandry and ESD are absent well (Romero and Pridgeon, 2009; Cribb et al., 2014). Based on the phylogenetic tree of Whitten et al. (2014) trees were rooted with *Polystachya*.

Ancestral State Reconstructions (ASRs) were conducted using phylograms obtained from ML inference (see above) and ultrametric trees (see below) following Cusimano and Renner (2014). Divergence time estimates were obtained with BEAST v. 2.1.3 (Bouckaert et al., 2014) using the CIPRES Science Gateway portal (Miller et al., 2010). Strict and uncorrelated lognormal molecular clock models, both with pure birth speciation models as recommended for species-level sampling (Bouckaert et al., 2014), were compared to

explore clock-likeness of the data. For calibrating the strict and relaxed clock model, there are unambiguously placed fossils available for Orchidaceae (Ramírez et al., 2007), but these are assigned to lineages very distantly related to Catasetinae (i.e., Agrostophyllinae, Dendrobiinae, Epidendreae, Malaxideae: Chase et al., 2015). Therefore, we assigned a normally distributed prior of 20 (±4 standard deviations) Ma to the crown group of Catasetinae obtained in the fossil-calibrated Orchidaceae chronogram of Chomicki et al. (2014). Secondary calibrations are best applied as normally distributed priors (Bouckaert et al., 2014). For each clock model, we ran two MCMC analysis with 20 million generations each, sampled every 1000th generation. Parameter convergence was confirmed using TRACER (available from http://beast.bio.ed.ac.uk/Tracer). Because fossil record is wanting for Catasetinae and closely related lineages (see above), and problems associated to secondary calibrations (see Forest, 2009; Graur and Martin, 2004), we do not discuss evolution of sexual systems in terms of absolute time estimates.

For coding, ESD (absence, state a; presence, state b) and sex distribution (bisexual, not protandrous flowers, state 0; protandrous, bisexual flowers, state 1; unisexual flowers, state 2) were personally observed for each species or obtained from the literature (Gregg, 1983; Romero and Nelson, 1986; Romero and Pridgeon, 2009). All species with ESD (state b) included in our sampling were simultaneously coded for unisexual sex distribution (state 2), because Catasetinae species having evolved ESD will produce always functionally unisexual flowers (see Introduction). Table S4 provides a list with all species sampled, their corresponding coding of each sexual system and the relevant references.

We carried out ML and Bayesian ancestral character reconstruction using the function *ace* of the package "APE" (Paradis et al., 2004), implemented in R (R Development Core Team, 2014) and the package *Multistate* of the software BayesTraits (Pagel, 1994), respectively. Under the ML approach, we fitted single (ER) and Asymmetrical Rate (ARD) models using a maximum clade credibility dated tree obtained from BEAST and a phylogram obtained from ML phylogenetic inference. Because ML reconstructions using both chronograms and phylograms yielded virtually the same output (results not shown), we used a chronogram (derived from BEAST analysis using a relaxed clock model, see Results section) instead of a phylogram, as *ace* function requires a fully bifurcating tree with positive branch lengths. To test the null hypothesis of "transition rates are equal between states of each character", a Likelihood Ratio Test (LTR) was performed to compare the likelihood obtained from the equal transition and the all-different transition models. Inferred character states of the best fitting model were plotted onto the dated phylogeny. Each trait was also inferred using a Bayesian approach and a set of ultrametric trees for comparison with the results obtained in the ML reconstruction and to take into account phylogenetic uncertainty. Character polarity with respect to protandry and ESD was investigated by estimating the rate coefficients of evolutionary transitions within states of each trait. For ASR of protandry, two models were fitted into Markov chains to determine which of the proposed models explains best the evolutionary scenario of the corresponding trait:

(1) A model M_1, in which all transitions are free (q_{01}, q_{10}, q_{12}, q_{21}, q_{ab}, q_{ba}, not restricted).
(2) M_2, in which transitions that involved direct switches from states 0 to 2 (i.e., q_{02}: bisexual, not protandrous flowers → unisexual) and *vice versa* (q_{20}), plus reversals from the sates 1 to 0 (q_{10}: bisexual, protandrous flowers → bisexual, not protandrous flowers), were set to 0 (i.e., not occurring).

Each of the models were compared to an equal transition rates model (M_0) via Bayes Factors (BF) tests, and the best model was chosen to reconstruct ancestral states. To obtain posterior probabilities and to infer character states at key nodes, independent reversible jump Markov chains (RJ MC) were ran for 30 million generations. The first 10 thousand iterations were discarded as burn-in, and the sampling fraction was set to every 1000th iteration. As rate transitions are not reliably known, gamma distributions ranging from 0 to 100 were chosen as priors. The RJ MC was executed using 5000 trees randomly sampled from those (~20 thousand) drawn by the Markov chain in the BEAST analysis. Random tree sampling was carried out in the software R, using the function *samples.trees* (available at http://coleoguy.blogspot.de/2012/09/randomly-sampling-trees.html). To better understand the parameters of the models visited by the RJ MC, the posterior distributions of the rate coefficients were plotted in the software R, using the function *plot.mcmc* of the package "CODA" (Plummer et al., 2006).

3. Results

3.1. Phylogenetic relationships and molecular clock dating within Catasetinae

We obtained new sequences of 77 Catasetinae species plus 5 outgroup taxa (a total of 154 new GenBank entries; see Table S1). The concatenated 'n' + 'cp' alignment was 2480 + 4321 bp in length and included 503 + 359 parsimony-informative positions (20% and 8%, respectively). Table 1 provides details of the aligned character matrices. Individual phylogenetic analyses of 'n' and 'cp' alignments provided high support for most nodes in trees (Fig. S1). After exclusion of chloroplast conflicting sequences (potential outliers detected by PACo are shown in Fig. S2, see Section 2), MP, ML and Bayesian trees of the 'n' and 'cp' data partitions recovered very similar, non-conflicting phylogenies (data not shown).

Fig. 2 shows the Bayesian majority-rule consensus tree as inferred from the concatenated alignment, with many nodes exhibiting high if not maximal support. Irrespectively of the method used, Catasetinae were monophyletic (100LBS, 100PBS, 1.00BPP) and included *Cyanaeorchis* (100LBS, 100PBS, 1.00BPP), *Grobya* (100LBS, 100PBS, 1.00BPP), *Galeandra* (100LBS, 100PBS, 1.00BPP) and the core Catasetinae (100LBS, 100PBS, 1.00BPP). All established generic lineages of core Catasetinae were strongly supported as well, with clearly resolved relationships: ((*Catasetum*, *Clowesia*), ((*Cycnoches*,*Mormodes*), (*Dressleria*))). Neither protandrous species, nor those exhibiting ESD, formed monophyletic groups. Instead, protandrous *Clowesia* and *Dressleria* were each closely allied to the ESD taxa *Catasetum* and *Cycnoches*, respectively, and *Mormodes* included a paraphyletic species group exhibiting protandrous flowers, from which a third ESD lineage arose.

3.2. Ancestral state reconstructions

Analysis of the log files generated under the relaxed clock model indicated that the concatenated 'n' + 'cp' alignment did not behave clock-like (coefficient of variation mean: 0.337). We

Table 1
Alignment characterisation.

Loci	Length (bp)	Parsimony informative sites (%)	Number of cells
ITS	925	273/29.6	81/82
ETS	549	170/31	42/82
Xdh	1006	60/6	33/82
matK	1679	155/9	17/82
ycf1	1738	126/7	30/82
*trn*S-G	904	78/8	27/82

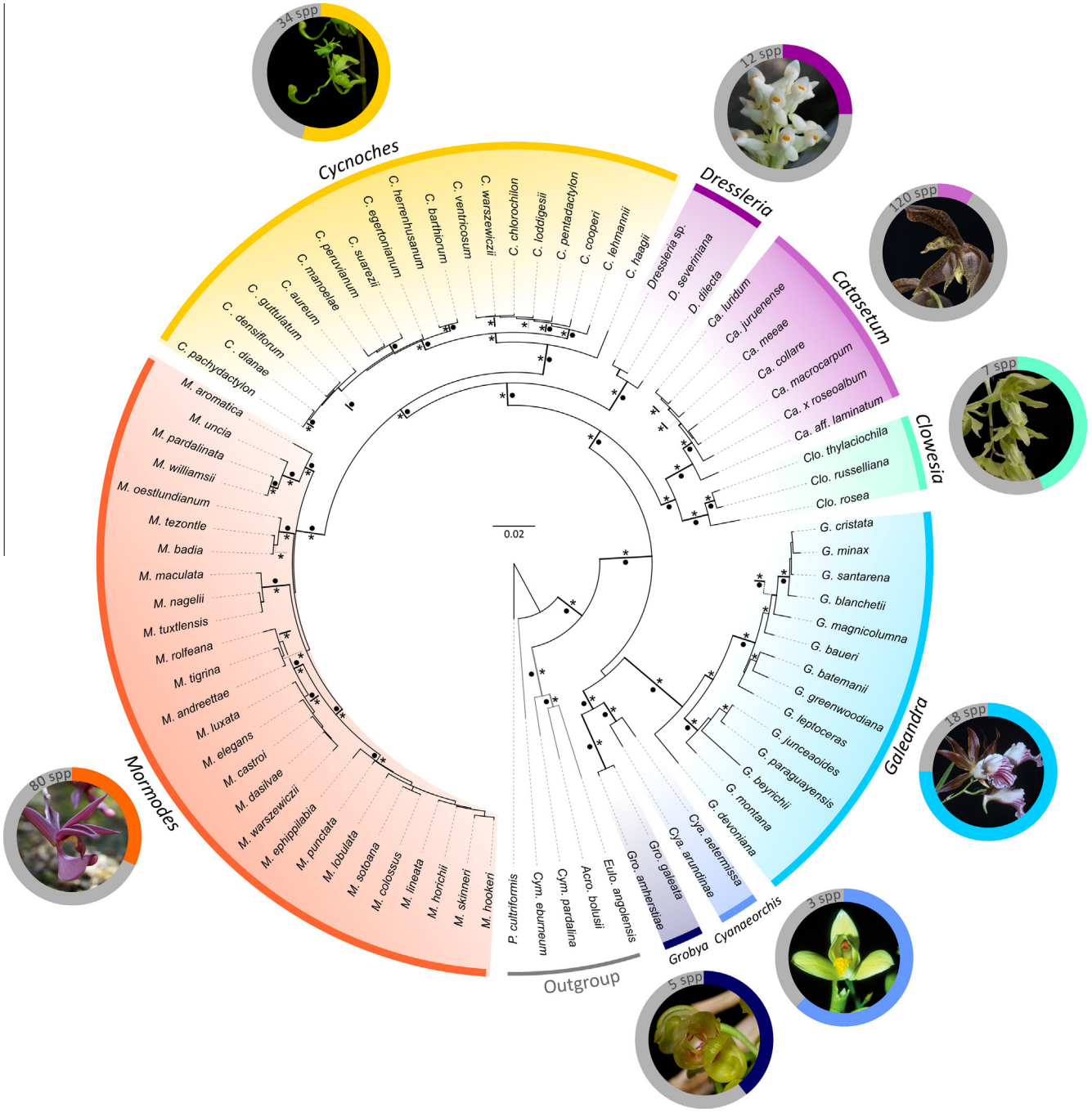

Fig. 2. 50% majority rule consensus tree inferred by Bayesian analysis showing strongly supported phylogenetic relationships of the Catasetinae. Likelihood (LBS), Parsimony Bootstrap Support (PBS) and Bayesian Posterior Probability (BPP) higher than 80%, 70% and 0.90 are indicated on the phylogeny with ticker branches, black circles and asterisks, respectively. Proportion of sampled (coloured portion of the circle) and extant species (numbers in grey inside the circle) of each genera of the Catasetinae are also provided. The percentages of sampled extant species for each lineage of the Catasetinae are also provided. Pictures: G. Gerlach, O. Pérez and J. Batista. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

therefore employed for ASR an ultrametric tree derived from BEAST analysis under a relaxed clock model. Maximum Likelihood ASR yielded comparable results using either phylograms or ultrametric trees (i.e., dated phylogeny) (results not shown). However, ultrametric trees were chosen (in favour of phylograms), as they are fully bifurcating, which is a requirement for ASR approaches such as the ML reconstruction implemented here. For the trait 'protandry' (Table 2), the Asymmetrical Rate Model (ARD) was favoured against equal rates evolutionary model (ER) as best fitting in ML approach and was employed for ASR inference. Nevertheless, no strong statistical support was obtained for the trait 'ESD' to reject the null hypothesis of equal rates, and we inferred the character using the ER model. For the ASR of 'protandry' under the Bayesian approach, the M_2 model (q_{02}, q_{20}, $q_{10} = 0$) was favoured over the M_1 model (all transitions free) via BF test. Table 3 provides harmonic means of the models tested and their corresponding BF scores.

A maximum clade credibility tree obtained from the BEAST analysis is presented in Fig. 3, with ancestral character states of ESD (left) and protandry (right) independently inferred under the

Table 2
Model testing (Equal Rate – ER, *vs.* Asymmetrical Rate Model – ARD) for ancestral state reconstruction under the ML approach by means of Likelihood Ratio Test (LRT).

Trait	States		Models		LRT
			Equal Rates (ER)	Asymmetrical Rates (ARD)	
ESD	Abscence (0)	Prescence (1)	−33.42951	−31.61023	3.63856
Protandry	Protandrous (0)	Not protandrous (1)	−41.57455	−30.68626	21.77658

Table 3
Model testing (Equal Rate – M_0 *vs.* all rates free – M_1 and q_{02}, q_{20}, $q_{10} = 0 - M_2$) for ancestral state reconstruction under the Bayesian approach by means of Bayes Factor (BF) test. Model chosen for ASR is highlighted in boldface.

Model	Harmonic mean	BF
M_0: Equal transition rates	−42.0350	–
M_1: All rates free (no restriction)	−80.8788	−77.688
M_2: q_{02}, q_{20}, $q_{10} = 0$	**−39.8809**	**4.308**

ML and Bayesian approaches at key nodes of the tree. Bayesian reconstructions yielded similar results to those obtained under the ML method for all the selected nodes, although not all reconstructions were statistically reliable (i.e., see standard deviations in Table 4).

With high confidence, the LCA of Catasetinae (node marked with a star in Fig. 3) bore bisexual, not protandrous flowers (100LBS, 1.00BPP) and did not exhibit ESD (95LBS, .65BPP). Such ancestral conditions were present also in the LCA of *Grobya* (node C), *Cyanaeorchis* (node B) and *Galeandra* (node O). The LCA of core Catasetinae (node N) bore bisexual, protandrous flowers, and ESD was likely not present. At nodes G (LCA of *Catasetum* + *Clowesia*) and M (LCA of *Dressleria* + *Cycnoches* + *Mormodes*), the protandrous condition was retained, but ESD was absent. At node L, the LCA of *Cycnoches* and *Mormodes* had protandrous flowers probably coupled with absence of ESD. In the respective LCAs of *Catasetum* (node F) and *Cycnoches* (node I) as only lineages consistently exhibiting ESD, unisexual flowers were present, and the derived status of ESD was thus confirmed.

Fig. 4 provides best model posterior distribution of rate coefficients for each transition between character states, together with their means, standard deviations, and the proportion of time each rate was assigned to a zero (Z) value. The transition rate coefficients obtained from the Bayesian approach reflects state reconstruction obtained with both ML and Bayesian RJ MC approaches. Posterior distributions of rate coefficients were assigned to two distinct classes of rates. (1) Rates almost never assigned to zero, and (2) rates assigned to zero more than 20% of chain iteration time. All transitions but one (see below) were associated to rate coefficients with positive values during most of the chain iteration time (rate class no. 1). Only the transition q_{ab} (representing switches from absence to presence of ESD) presented zero values more than 20% of the chain iteration time (rate class no. 2).

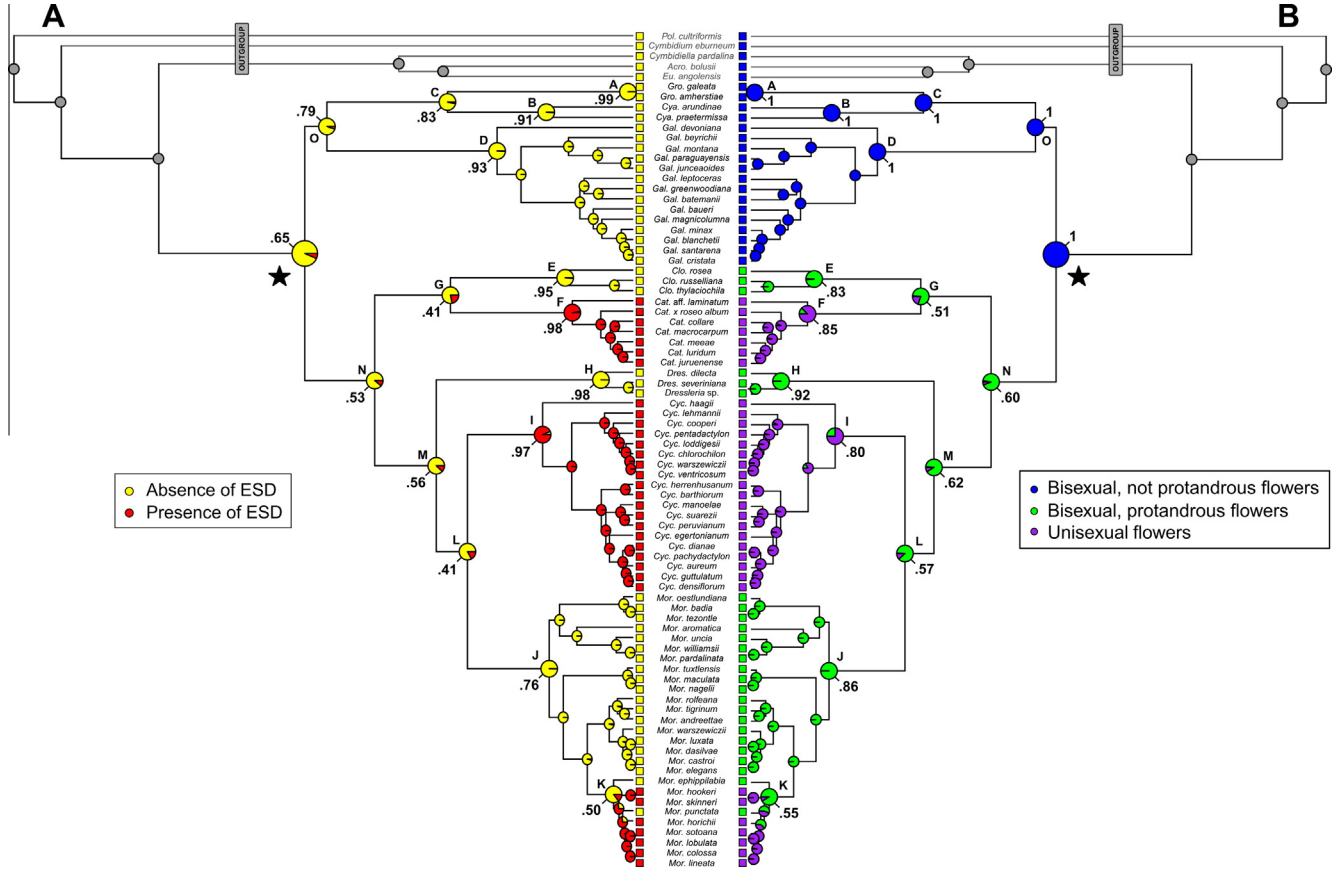

Fig. 3. Maximum Likelihood optimizations under an Asymmetrical Rate Model (ARD) of the sexual systems of (A) environmental sex determination (ESD) and (B) protandry of Catasetinae on a BEAST maximum credibility clade phylogeny (ages not shown). Posterior probabilities of each state occurrence obtained from Bayesian reconstructions are shown at key node at the phylogeny (labelled with letters, see Table 4 for detailed values). The LCA of Catasetinae is indicated with a black star.

Table 4
Reconstruction of trait evolution (Pr = protandry, ESD = environmental sex determination), on selected nodes of the Catasetinae phylogeny using a RJ-MCMC approach. Standard deviations of posterior probabilities are also provided.

Node	*P*(Pr = 0)	SD	*P*(Pr = 1)	SD	*P*(Pr = 2)	SD	*P*(ESD = 0)	SD	*P*(ESD = 1)	SD
MRCA	1	0	0	0	0	0	0.6500	0.3700	0.353	0.374
A	1	0	0	0	0	0	0.9900	0.0010	0	0.002
B	1	0	0	0	0	0	0.9100	0.0850	0.087	0.085
C	1	0	0	0	0	0	0.8349	0.1250	0.165	0.125
D	1	0	0	0	0	0	0.9372	0.0720	0.063	0.072
E	0.0020	0.0036	0.8344	0.1534	0.1637	0.1534	0.9511	0.0530	0.049	0.053
F	0.0001	0.0003	0.1479	0.1366	0.8521	0.1367	0.0156	0.0310	0.984	0.031
G	0.0031	0.0051	0.5589	0.1090	0.4380	0.1093	0.4187	0.2400	0.581	0.249
H	0.0005	0.0010	0.9228	0.1139	0.0767	0.1138	0.9858	0.0210	0.014	0.021
I	0.0001	0.0004	0.1966	0.1445	0.8033	0.1447	0.0241	0.0410	0.976	0.042
J	0.0002	0.0004	0.8622	0.1655	0.1377	0.1655	0.7590	0.4100	0.241	0.413
K	0.00002	0.0001	0.5522	0.2867	0.4477	0.2867	0.5035	0.3800	0.497	0.382
L	0.0015	0.0023	0.5797	0.1240	0.4188	0.1241	0.4140	0.2400	0.586	0.246
M	0.0023	0.0042	0.6281	0.1517	0.3696	0.1524	0.5610	0.3200	0.439	0.321
N	0.0020	0.0039	0.6034	0.1491	0.3947	0.1498	0.5321	0.3000	0.468	0.308
O	1	0	0	0	0	0	0.7940	0.1400	0.206	0.141

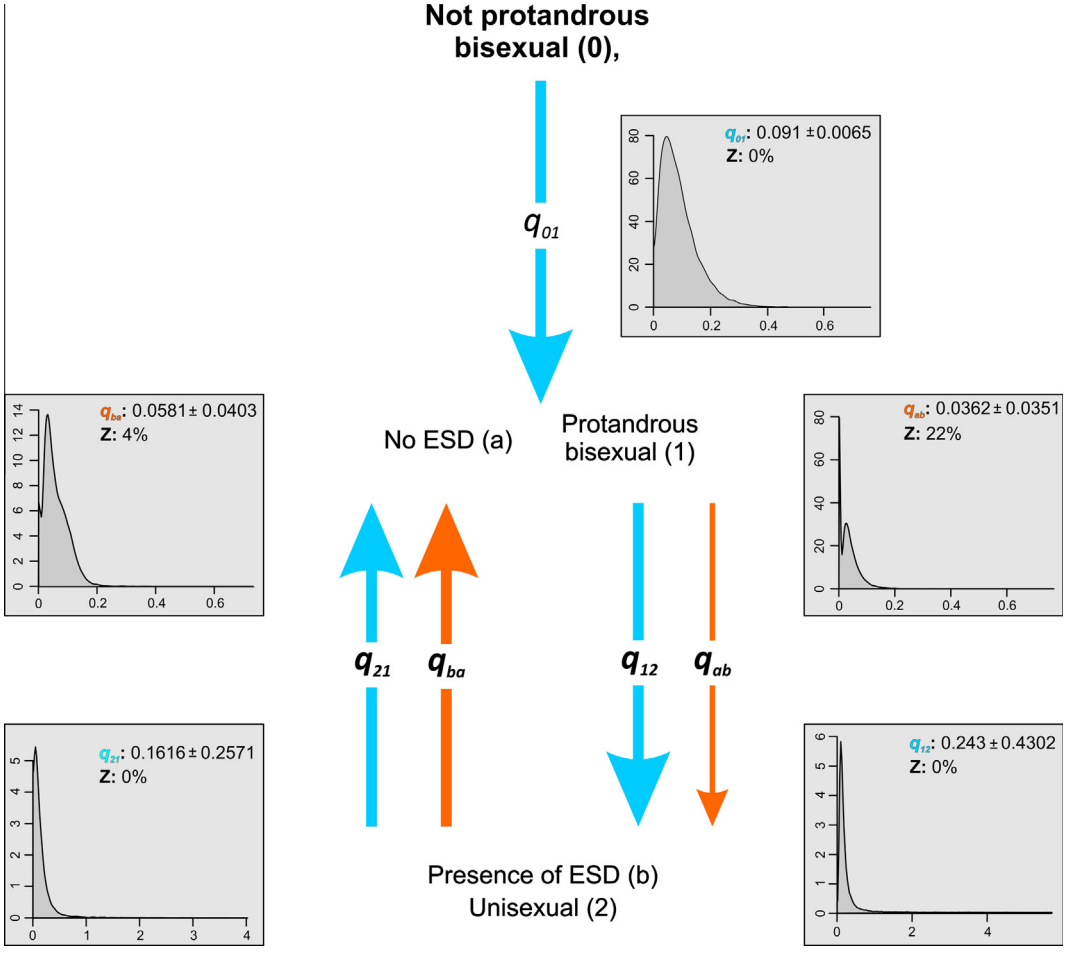

Fig. 4. Posterior distribution of rate coefficients and their respective mean and standard deviation obtained from 30,000 observations sampled from 50 million iterations of a RJ MC and their corresponding flow chart indicating the most likely evolutionary scenario of protandry and ESD in the Catasetinae. Thick arrows correspond to transition rates whose posterior probability were seldom or never assigned to zero (rate class no. 1). The ancestral character state of the LCA of Catasetinae is shown in bold, as inferred from ML and Bayesian approaches.

4. Discussion

Previous phylogenetic studies of Catasetinae (Batista et al., 2014; Chase and Pippen, 1990; Pérez-Escobar et al., 2015; Pridgeon and Chase, 1998; Romero, 1990; Stern and Judd, 2001; Whitten et al., 2014) have included a few species of the established generic lineages only, and the resulting trees have been thus not representatively sampled. Our analysis of a larger sampling of species, including all established generic lineages of Catasetinae, confirms their monophyly and also receives strong statistical support for almost all their internal relationships. Therefore, it provides a robust phylogenetic framework for the rigorous study

of sexual system evolution and other traits of interest (such as pollination syndromes) in this lineage.

Few studies have addressed the evolution of mating systems in orchids (e.g., Pleurothallidinae: Borba et al., 2011), and they have drawn conclusions based solely on extensive observations without test using a well resolved phylogenetic tree as basic tool. For core Catasetinae (as circumscribed in the Introduction), either protandry or unisexuality (i.e., ESD) has been considered the apomorphy and thus the ancestral state for the LCA's descendants (Romero, 1990). Our ASRs favour the assumption, in which core Catasetinae initially have bisexual, protandrous flowers (i.e., without ESD). Protandry has evolved independently several times, also in Orchidaceae across only distantly related lineages (e.g., Catasetinae and Stanhopeinae, Cranichidinae, Goodyerinae, Manniellinae and Spiranthinae, Neottieae and Satyriinae: Ackerman, 1977; Darwin, 1877; Jersáková and Johnson, 2007; Singer and Sazima, 2001; Salazar et al., 2002; Singer and Koehler, 2003). However, we demonstrate that protandry has a single origin during the early evolutionary history of Catasetinae, with several subsequent losses in descendant lineages.

Environmental sex determination is an exceedingly rare trait among Orchidaceae, which has exclusively developed in some of the core Catasetinae and specifically in *Catasetum*, *Cycnoches* and some species of *Mormodes*. It is thus a striking result of our study to show three independent origins of ESD within core Catasetinae, always evolved from a protandrous ancestor. In other plant lineages with this character (e.g., *Acer*, Aceraceae; *Elaeis* Jacq., Arecaceae; *Gurania* (Schltdl.) Cogn. and *Psiguria* Neck. Ex Arn., Cucurbitaceae: Renner et al., 2007), ESD has a single origin only and is thus homologous. Moreover, gain of ESD is linked to loss of protandry in core Catasetinae, and this is also supported for all key nodes of our trees with confidence under all ASR approaches executed. Thus, prior gain of protandry is the necessary prerequisite for gain of ESD.

Detailed studies in several plant lineages (e.g., *Fuchsia* L., *Hebe* Comm. ex Juss.: Atsatt and Rundel, 1982; Delph, 1990) have shown that labile sexual expression is involved in transitions to and out of dioecy (Delph and Wolf, 2004). Although plants of core Catasetinae exhibiting ESD are not fully dioecious (mixed inflorescences with staminate and pistillate flowers are produced at least occasionally; Fig. 1A and B), there is a strongly biased production of inflorescences bearing solely unisexual flowers in natural populations (e.g., Romero and Nelson, 1986; O.A. Pérez-Escobar, *pers. obs.*). Therefore, it appears that ESD might be indeed an intermediary stated between the evolution of monoecy and dioecy, as stated by Delph and Wolf (2004).

The transition rate coefficients provide a likely evolutionary scenario for sexual systems such as protandry and ESD in Catasetinae (Fig. 4). Transition rates leading from the state of bisexual, non-protandrous flowers to bisexual, protandrous flowers (q_{01}), is in line with ASR of core Catasetinae's LCA inferred under alternative approaches. Additionally, it supports the single gain of protandry in the LCA of core Catasetinae (node N in Fig. 3) because transition rate of reversions back to non-protandrous, bisexual flowers (q_{10}) is 0 (the model of choice does not allow such reversals). Alternate models, in which this transition is allowed, are less-fitted as inferred with BF (see Table 3 for model comparisons). This is also reflected in our ASRs, since the lineages presenting protandrous, bisexual flowers (i.e., *Clowesia*, *Dressleria* and some species of *Mormodes*) have retained this condition and therefore, no reversals to bisexual, non-protandrous flowers are observed at descendant nodes. Transitions leading to unisexual flowers (q_{12}) with ESD (q_{ab}) also reflect the multiple gains of unisexuality and ESD recovered in other reconstruction approaches (e.g., nodes F and I), indicating that this sexual system might be in fact a homoplasious character.

Putative transitions towards secondary loss of ESD (q_{ba}) and reversals towards bisexual, protandrous flowers (q_{21}) may also be a result of phylogenetic uncertainty that has been taken into account when inferring this trait using a set of trees under a Bayesian approach (see Section 2). Interestingly, gains of ESD (q_{ab}) may have occurred slower (transition assigned to rate class no. 2) than losses of this trait (q_{ba}), supporting the assumption that complex traits are indeed more easily lost than gained (Pagel, 2006; Barret, 2013). Additionally, the repetitive gains and reversals of ESD and unisexuality might also indicate that presence of such traits apparently does not represent an evolutionary advantage for these lineages.

In conclusion, evolution of sexual systems implies that non-protandrous, bisexual flowers without ESD are the ancestral character state in Catasetinae (as it probably can be stated generally for angiosperms). Protandry has been gained once by the LCA of core Catasetinae and subsequently lost three times independently, always coupled with gains of ESD. In addition, ESD is a homoplasious character, whereas protandry is an apparently inherited, conserved trait (as observed in *Catasetum*, *Cycnoches* and *Mormodes* for ESD and the core Catasetinae for protandry). The multiple inferred origins of ESD is contrastingly different from other angiosperms, for which trait homology has been shown (Renner et al., 2007). Bertin and Newman (1993) suggested that protandry is a "phylogenetic relic" (conserved character) in lineages, where other specialised systems favouring outcrossing have evolved. As inferred from our ASRs, protandry is the prerequisite (a state retained by *Clowesia*, *Dressleria* and some species of *Mormodes*) for unisexual flowers with ESD. Finally, to determine whether ESD and protandry are truly correlated sexual systems, research must clarify the underlying genetic mechanisms controlling both systems and their corresponding driving evolutionary forces.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ympev.2015.11.019.

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Chapter 7

HISTORICAL BIOGEOGRAPHY OF *CYCNOCHES* (CATASETINAE): THE IMPROBABLE JOURNEYS OF SWAN ORCHIDS ACROSS THE ANDES

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HISTORICAL BIOGEOGRAPHY OF *CYCNOCHES* (CATASETINAE): THE IMPROBABLE JOURNEYS OF SWAN ORCHIDS ACROSS THE ANDES

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Abstract:

Aim: The Andean uplift is one of the major orographic events in the New World, responsible for the diversification of numerous Neotropical plant lineages. Despite its importance for historical biogeography, the specific role in geological times as a dispersal barrier between South and Central American lowland lineages is still poorly understood. The rare swan orchids (*Cycnoches*, Catasetinae) comprise ca. 34 epiphytic species distributed in altitudes below 800 m in lowland and pre-montane forests of Central and South America. Here we study the biogeographical history of the swan orchids to better understand the impact of the Andean uplift on the diversification of Neotropical lowland centred lineages.

Location: northern South America and Central America.

Methods: Three nuclear loci and two chloroplast DNA regions were sequenced for 23 species representing the currently known distribution of *Cycnoches*. Nine outgroup taxa distributed in different tropical regions were also included in our sampling to ensure unbiased ancestral area inference. Absolute ages were inferred under strict and relaxed molecular clock models, and ancestral areas were estimated under several models in a Maximum Likelihood framework.

Results: The last common ancestor of *Cycnoches* may have lived in the Amazonian region ca. 6 mya and dispersed towards the Choco region and Central America in multiple migration events. Stochastic mapping revealed that speciation despite sympatric occurrence played an important role on shaping the current range distribution of *Cycnoches* species.

Main conclusions: The Amazonian lowland is an important area of origin for epiphytes such as *Cycnoches*. Multiple migrations from the Amazonian region to Central America (and later also back) have occurred well after major mountain building periods. The Andes thus do not appear as an effective barrier for lineages such as orchids having a great potential for dispersal dynamics because of the very light, anemochorous seeds.

Key words: ancestral area, *Cycnoches*, model testing, molecular clock, orchids, anemochory

Introduction

Neotropical landscapes have ever drawn the attention of ecologists, botanists and more recently molecular biologists (e.g., Humboldt, 1820; Darwin, 1846; Antonelli et al., 2010; Batalha-Filho et al., 2014) because of the rich biodiversity and their remarkable levels of endemism (Jaramillo et al., 2006; Antonelli & Sanmartín, 2011). The combination of molecular phylogenies with evidence from distribution and the fossil record has enlightened different biotic and abiotic factors responsible for diversification in the Neotropics (Antonelli et al., 2009; Hoorn et al., 2010; Bacon et al., 2015). However, biogeographical studies applying such approaches are available for few Neotropical plant clades only (e.g. Antonelli et al., 2009, 2010; Luebert et al., 2011; Chacón et al., 2012; Bacon et al., 2013). They have demonstrated the importance of geological processes such as Andean mountain uplift and establishment of the Isthmus of Panama for the evolution of Neotropical plants.

One of the most relevant abiotic processes in the diverse geological history of the Americas is the rise of the Andes (Luebert et al., 2011). Andean mountain building was driven by plate tectonic re-adjustments that started during the Paleogene and continued until the Pliocene (Hoorn et al., 2010). Fossil record (e.g., palynological data: Jaramillo et al., 2006) and geological data (e.g., isotope measurements: Ghosh et al., 2006; sediment loads, apatite fission-track data: Hoorn et al., 2010) indicate that the Andean uplift was a partially constant process alternating with discrete periods of intensified mountain building.

Newly formed mountain ranges may had an enormous impact on the adjacent Amazonas landscape and the inhabiting organisms by transformation of its drainage systems (Hoorn et al., 1995), but also in local weather by forming the only barrier to atmospheric circulation in the region (Gregory-Wodzicki, 2000). More importantly,

Andean uplift has provided a great variety of new, partly very fine-scaled habitats (Vuilleumier, 1971) as well as physical-ecological barriers in greater dimensions. The efficiency of the Northern Andes as migration barrier has been also shown for Central American woody species of *Sapranthus* Seem. and *Tridimeris* Baill. (Annonaceae), which are animal dispersed (Janzen & Martin, 1982) and confined to the Colombian Pacific coast (Pirie et al., 2006).

Some studies provide solid evidence for the important role of Andean uplift in diversification of several geophyte highland plant groups (e.g., *Lupinus* L.: Hughes & Eastwood, 2006; *Bartsia* L.: Uribe-Convers & Tank, 2015), but the impact of such orographic processes for the lowland flora is still poorly understood (Antonelli et al., 2009). Nevertheless, few available studies for lowland geophyte plant clades have shown that the Andean uplift indeed has acted as a physical barrier. In Rubiaceae, for instance, the LCA of the sister clades Cinchoneae and Isertieae is inferred to have had a lowland distribution ~42 mya. Diversification of Isertieae may have taken place in Amazonian lowland forests during Middle to Late Miocene, while the LCA of Cinchoneae is considered to have diversified in higher altitudes in Northern and Central Andes (Antonelli et al., 2009). The diversification of these lineages remarkably coincides with mountain building periods of the Eastern Cordillera in Northern Andes (Hoorn et al., 1995).

The question remains whether Andean uplift has indeed been an abiotic barrier to migrate for epiphytic lineages such as lowland orchids and bromeliads. Epiphytic diversity is dramatically greater in the Neotropics than in any other tropical region of the world (Kreft et al., 2004), being as twice as high than, for instance, in Australasia (Gentry & Dodson, 1987). Several traits shared by Neotropical epiphytic taxa, related all with their reproductive biology, might explain such overwhelming difference in diversity. One of the

most prominent shared traits are the lightness and very small size of the propagules (e.g., bromeliads, ferns, orchids, *Utricularia* L.) occasionally with highly elaborated epidermis (Gentry & Dodson, 1987). The capability of dust-like seeds for anemochory may indicate their potential for longer distance dispersals compared to other plant clades with propagules rather locally dispersed by animals (e.g., Araceae: Nauheimer et al., 2012). Nonetheless, whether lowland epiphyte lineages have been able to disperse across large distance and cross geographic barriers such the Andes is largely unknown, last but not least because of a general lack of representatively sampled phylogenies available for such clades.

Several anemochorous plant lineages (e.g., *Begonia* L., bromeliads) span across the Neotropical region, many of which are restricted to lowland elevations. One such example is the orchid tribe Cymbidieae comprising ca 3900 species that are distributed mostly in the Neotropics (but with few representatives in the Old World Tropics: Pridgeon et al., 2009). Among the Neotropical taxa of Cymbidieae is the swan orchid *Cycnoches* Lindl., and the members are known for the striking sexual dimorphism (Fig. 1 A-C; Pérez-Escobar et al., *in press*). Molecular phylogenetic and morphological studies conducted to date confirm the inclusion of *Cycnoches* in Catasetinae (Chase & Pippen, 1990; Romero, 1990; Stern & Judd, 2001), as sister group of *Mormodes* Lindl. (Batista et al., 2014; Whitten et al., 2014; Pérez-Escobar et al., *in press*.).

Cycnoches encompasses 34 species (Carr, 2012) that are distributed from Southern Mexico to Central Brazil and Bolivia. They commonly inhabit tropical wet forests and lowlands, ranging from 0 to 800 m., although sporadically, herbarium records push the altitudinal range limit to 1200 m. Unlike all other Orchidaceae, flowers of *Cycnoches* and other members of the Catasetinae such as *Mormodes* and *Catasetum* Rich ex Kunth are sexually dimorphic, and a single plant is able to exhibit functional staminate or pistillate

flowers (Fig 1 D-G) (Gerlach, 2007). *Cycnoches* can be further distinguished from other Catasetinae by having an elongate column in functionally staminate flowers (Gerlach & Pérez-Escobar, 2014) (Fig. 1D).

Swan orchids have attracted the attention of several prominent botanists including Charles Darwin (1877), but doubts still surround their taxonomy. Previous phylogenetic studies have included no more than three species of *Cycnoches* (Chase & Pippen, 1990; Romero, 1990; Pridgeon & Chase, 1998; Batista et al., 2014; Whitten et al., 2014) and hence, the internal phylogenetic relationships are elusive to present. An evidence of the intricate taxonomy of the lineage is the existence of species complexes including extremely variable morphological species that are often difficult to determine. One such example is the *Cycnoches egertonianum* species complex (Romero and Gerlach, *in press*) that encompasses ten entities distributed from southern Mexico to Southern Panama and Colombia (Fig. 3; see Gerlach and Pérez, 2014 for a detailed description on the species complex). Anyhow, the lack of a solid, internal phylogeny of *Cycnoches* has precluded researchers to address specific questions concerning the role of Andean uplift in the biogeographic history of this lineage.

In this study, we use three nuclear and two chloroplast loci from 24 of 34 known species to infer internal phylogenetic relationships of *Cycnoches*. Based on a solid phylogenetic framework, we use Ancestral Area Estimation (AAE) analysis to test whether Andean uplift has influenced clade diversification within *Cycnoches*, as observed in other plant lineages such as Rubiaceae (Antonelli et al., 2009) and Annonaceae (Pirie et al., 2006). By determining the putative area and geological time of origin, we aim to provide an evolutionary scenario for *Cycnoches* with the potential to explain diversification also in other plants group with diverse epiphytic growth forms.

Material and methods

Taxon sampling, DNA sequencing and phylogenetic analysis

Table S1 of Appendix S1 provides species names, geographic origins, voucher specimens and GenBank accession numbers of sequences included in phylogenetic analyses. Genomic DNA was extracted from herbarium and fresh leaf material with the NucleoSpin® plant kit (Macherey-Nagel; Düren, Germany), following the manufacturer's protocol. We amplified and sequenced nuclear (consistently referred as 'n' henceforth) ribosomal external and internal transcribed spacers (ETS and ITS, respectively), a fragment of the 'n' gene *Xdh*, a ~1500 bp fragment of the chloroplast (henceforth referred as 'cp') gene *ycf*1, as well as the 'cp' *trn*S–*trn*G intergenic spacer. Amplification settings and sequencing primers used for ITS, ETS, *Xdh*, *trn*S–*trn*G and *ycf*1 are specified in Tab. S2 of Appendix S1. Amplified PCR products were purified with the ExoSap clean-up kit (Fermentas; St. Leon-Rot, Germany), and sequencing reactions were run on an ABI 3130 capillary sequencer (Thermo Fisher Scientific; Waltham USA) following the manufacturer's instructions. Sequence editing was carried out using Geneious software v. 7.1.7 (Biomatters Corporation; Auckland, New Zealand).

Loci were aligned separately using MAFFT version 7.1 (Katoh & Standley, 2013). For 'n' ribosomal RNA loci and 'cp' *trn*S–*trn*G spacer, secondary structure of molecules were taken into account (i.e., the --qINSi option). Congruence between 'n' and 'cp' data sets was assessed following Pérez-Escobar et al. (2015), using PACo application (Balbuena et al., 2013). The procedure is now available as a pipeline (http://www.uv.es/cophylpaco/) and was also employed to identify outlier Operational Terminal Units (OTUs) from the 'cp' data set that were found to be conflicting with the 'n' data set (potential outliers detected by

PACo are shown in Fig. S1 in Appendix S1). After removing outliers, matrices of each locus were re-aligned and concatenated.

Phylogenetic analyses of separate and concatenated loci were carried out under Maximum Likelihood (ML) and Bayesian criteria using the GTR+Γ substitution model (with four categories). For this purposes, software programs RAxML-HPC version 8.2.4 (Stamatakis, 2014) and MrBayes version 3.2.2 (Ronquist et al., 2012) were used at the CIPRES Science Gateway computing facility (Miller et al., 2010). Bayesian inferences were carried out with two independent runs of four Markov chain Monte Carlo (MCMC) analyses with 20 million generation each, sampled every 1000^{th} generation and using default prior settings. Statistical support values (BPP: Bayesian posterior probabilities, LBS: ML bootstrap support) were drawn on the best scoring ML tree.

Molecular clock dating

Divergence time estimates were conducted using BEAST v. 2.1.3 (Drummond & Bouckaert, 2014) at the CIPRES Science Gateway computing facility and a concatenated 'n'-'cp' subset of the data obtained after PACo analysis (see above). Strict and uncorrelated lognormal molecular clock models, both with pure birth speciation models as recommended for species level sampling (Bouckaert et al., 2014), were compared to explore clock-likeness of the data. For calibrating the relaxed clock model, there are fossils available unambiguously to be placed for Orchidaceae (Ramírez et al., 2007), but these are assigned to lineages very distantly related to *Cycnoches* (i.e., *Dendrobium* Sw., *Earina* Lindl., both Vandeae). Secondary calibrations are therefore best applied as normally distributed priors (Bouckaert et al., 2014), for which we used 20 and 27.1 (±4 and ±6 standard deviation) mya. Such values corresponded to the crown group of Catasetinae and to the root of our

199 trees (LCA of Eulophiinae + Catasetinae), respectively, as obtained from fossil-calibrated
200 Orchidaceae chronogram of Chomicki et al. (2014). For strict molecular clock calibration,
201 we placed only a single constraint at the tree root (27.1 mya ±6 standard deviation). For
202 each clock model, we ran two MCMC analysis with 20 million generations each, sampled
203 every 1000th generation. Parameter convergence was confirmed using TRACER (available
204 from http://beast.bio.ed.ac.uk/Tracer).

205 *Ancestral Area Estimation*

206 Species ranges were coded from the literature (Carr, 2006; Romero, 2009) and from
207 herbarium specimens (AMES, COL, F, M, MO, SEL, US). Distribution data was also
208 obtained from own field observations. Distribution maps of the orchids under investigation
209 (Fig. S2 of Appendix S1) as well as distributions observed in other plant lineages (e.g.,
210 Rubiaceae: Antonelli et al., 2009) allowed for distinction of three main distribution areas:
211 1) Central America (comprising southern Mexico through Panama); 2) Amazonia,
212 including pre-montane forests (encompassing lowlands and montane forest below 1200 m
213 in Colombia, Ecuador, Peru, Brazil, Venezuela, Guyana, Suriname and French Guiana:
214 Antonelli et al., 2009). 3) Chocó (comprising lowlands below 500 m of the western Andes
215 in Colombia and Ecuador).; 4) Africa (distribution range of *Eulophia petersii* (Rchb.f.)
216 Rchb.f., outgroup taxon chosen for rooting purposes). A map with coded distribution areas
217 is provided in Fig. 3 (inset), and all species under investigation were assigned to one of
218 those regions.

219 For AAE in *Cycnoches*, we used the package BioGeoBEARS (Biogeography with
220 Bayesian and Likelihood Evolutionary Analysis in R script: Matzke, 2014) as implemented
221 in the free software R (R Development Core Team, 2014). Unlike previously provided

222 applications such as LAGRANGE: Ronquist, 1997; Ree & Smith, 2008), BioGeoBEARS
223 evaluates altogether several processes that were taken into account to explain today's
224 observed distributions (i.e., range expansions, local extinctions, founder-event speciation,
225 vicariance, and speciation despite sympatry) in a joint statistical framework. It is therefore
226 capable of model testing and hence determines which process fits best the geographical and
227 phylogenetic data for any particular clade (Matzke, 2013). In order to test whether the
228 Andes was an effective isolative barrier in *Cycnoches*, no dispersals constrains were
229 defined for AAE approaches. In addition, the maximum number of estimated areas at nodes
230 were set to two, following the maximum number of areas occupied by extant species coded
231 in our phylogeny. In order to estimate the mean number of migrations, dispersals, local
232 extinctions and speciation events despite sympatry from our phylogeny, we used
233 Biogeographical Stochastic Mapping (BSM) (Matzke, 2014) under the best fitting model,
234 as likewise implemented in the package BioGeoBEARS.
235
236

Results

Phylogeny of Cycnoches

In this study, 80 sequences were newly generated (Appendix S6). Our phylogeny comprised 22 out of 34 described species. Tab. S3 of Appendix S1 provides detailed alignment descriptions. The concatenated 'n' alignment was 2395 bp and included 310 parsimony informative sites, while the concatenated 'cp' alignment was 2419 bp and comprised 171 parsimony informative positions. Individual ML and Bayesian analysis of each partition recovered virtually the same topology (data not shown), and they provided maximal support for the monophyly of *Cycnoches*. Nevertheless, independently derived concatenated 'n' and 'cp' phylogenies revealed conflicting and highly supported phylogenetic placements (see below). Fig. S3 of Appendix S1 shows trees individually derived from concatenated 'n' and 'cp' datasets together with outlier OTUs retrieved by PACo method (see *materials and methods*; Fig. S1).

Figure 2 provides the best scoring ML tree inferred from non-conflicting, concatenated 'n' and 'cp' datasets showing the internal phylogenetic relationships of *Cycnoches*. Virtually, all backbone nodes of the phylogeny were highly, if not maximally supported by LBS and BPP values. *Cycnoches* segregated into three main lineages (clades A, B and C), each of which included species with similar morphological traits (Fig. 2). All accessions of *Cycnoches haagii* Barb.Rodr. (clade A) were sister group of the remaining species of *Cycnoches* placed into clades B and C. The *Cycnoches egertonianum* species complex (five taxa here sampled: *C. amparoanum* Schltr., *C. egertonianum* Bateman, *C. densiflorum* Rolfe, *C. guttulatum* Schltr., *C. pachydactylon* Schltr., *C. rossianum* Rolfe) was recovered as polyphyletic. In contrast, it clustered in two strongly supported lineages within Clade C (Fig. 2). The first clade comprised C. *egertonianum* var. *egertonianum*, *C.*

egertonianum var. *viride* Lindl. and *C. rossianum* occuring in southern Mexico, southeastern Costa Rica and north eastern Panama. The remaining clade included *C. densiflorum*, *C. guttulatum* and *C. pachydactylon*, which are distributed from west to south east of Panama and Northern Colombia.

Molecular clock dating

Estimations of absolute ages of main divergence events under strict and relaxed clock models are shown in Table 1. Such estimations slightly differed under these models and across all tree nodes (dated phylogenies inferred under both clock models are shown in Figure S4–S5 of Appendix S1). Analysis of the log file produced by dating analysis under the relaxed clock yielded a coefficient of variation value (CV) of 0.245 (ESS value of 2489). A chronogram showing absolute ages estimated under a relaxed clock is presented in Figure 3. *Cycnoches* and *Mormodes* shared a common ancestor during the middle Miocene (11 mya). Diversification of *Cycnoches* took place around 6 mya during the late Miocene. The split between clades B and C of *Cycnoches* occurred somewhere during late Pliocene (3.93 mya). Clades B and C together were estimated to 2.29 and 1.82 mya, respectively. The split between the two lineages of the *Cycnoches egertonianum* species complex was estimated to 1.5 mya (node L, Fig. 3) during the Pleistocene.

Ancestral Area Estimation

Table 2 provides model test statistics for all models employed in AAE. The best fitting model for our phylogenetic and geographical data was the Dispersal and Vicariance model (DIVA), including the founder- event speciation (free parameter *j*, -32.72 Ln *L*, Tab. 1) as inferred in BioGeoBEARS. The LCA of *Cycnoches* originated in Amazonia (Fig. 4, Node G) and also later, descendant species corresponding to nodes H, I and J, respectively, may have been migrated towards that region. Several long-distance dispersal events from

285 Amazonia to Choco and Central America could be stated. For example, two lineages
286 (namely *C. lehmannii* Rchb.f., *C. barthiorum* G.F.Carr & Christenson and *C.*
287 *herrenhusanum* Jenny & G.A.Romero) independently colonised the Choco region, the
288 former from Amazonas region whereas the two latter taxa likely from Central America
289 (Fig. 3). Moreover, a nested lineage in clade C (Fig. 3, node L) colonised Central America
290 and subsequently diversified here (eight species). Nevertheless, the LCA' ancestral area of
291 such clade and its corresponding Chocoan based sister clade was ambiguously
292 reconstructed (Fig. 3), and therefore it is unknown whether colonization occurred from
293 Amazonas region. Independent colonisation from Amazonia region to Central America was
294 also observed in two members of clade B, namely *C. ventricosum* Bateman and *C.*
295 *warszewiczii* Rchb.f..

296 Count events of BioGeoBEARS parameters estimated by BSM method under the
297 DIVA and DIVA+J model are presented in Table 3. Under the DIVA+J process, the most
298 relevant causes for *Cycnoches* speciation were within region (mean 16.34, *y* parameter) and
299 founder-event speciation processes (mean 6.62, *j* parameter). In contrast, under DIVA
300 model, dispersal (*d* parameter), sympatry and vicariance (*v* parameter) were the most
301 frequent phenomena (mean 6.74, 16.16 and 6.84, respectively).

302

303

Discussion

Supported phylogenetic relationships within Cycnoches

Previous phylogenetic studies about Catasetinae have included not more than three species of *Cycnoches* (Chase & Pippen, 1990; Romero, 1990; Pridgeon & Chase, 1998; Batista et al., 2014; Whitten et al., 2014). Our larger, being the most representative sampling of *Cycnoches* available at present confirms and strongly supports its monophyly inferred from both nuclear and chloroplast sequence data sets. Missing taxa in our sampling belonged mostly to Amazonian species, from which only inaccessible type collections are known (e.g., *C. carrii* Christenson, *C. jarae* Dodson & D.E.Benn.). Morphologically, the monophyly of *Cycnoches* is corroborated by the presence of a unique arched, elongated, slender column in staminate flowers.

Because of the limited taxon sample of previous studies, the internal relationships of *Cycnoches* have remained unresolved as well. Our phylogenetic inferences strongly support its division into three main lineages (Clade A, B and C: Fig. 3). This result conflicts with Rolfe's (1909) traditional infrageneric classification into sections *Cycnoches* (morphological similarity between staminate and pistillate flowers) and *Heteranthae* (with dissimilar staminate and pistillate flowers). Rather, analysis of our nuclear data set provides strong evidence for two independent origins of strong sexual dimorphism in *Cycnoches*, firstly within clade B (i.e., LCA of *C. cooperi* Rolfe and *C. pentadactylon* Lindl.) and secondly in clade C (Fig. 2).

Biogeographical history and diversification of Cycnoches

Our study provides a solid phylogenetic framework for divergence time estimation and ancestral area reconstruction in *Cycnoches*. The following discussion is focused on ages obtained under the relaxed clock model (CV value of 0.245, see *results*) fitting best to our

data as inferred from log file analysis (Drummond and Bouckaert, 2014). Central America has been considered the most likely region of origin for *Cycnoches*, followed by accelerated species diversification particularly in Panama lowland forests (Romero and Gerlach, *in press*). However, this scenario is rejected by our AAR, as it supports an Amazonian origin of *Cycnoches*. The LCA may have lived in the late Miocene (~6 mya, Fig. 3), well after one of the most intense Andean mountain building events (ca. 12 mya; Hoorn et al., 2010).

The Amazonas is inferred the most important source area for *Cycnoches*, as all dispersals towards the Choco region and Central America have occurred exclusively from that region. Such range expansion events have taken place fairly recently, from middle to late Pleistocene. Additionally, a single, very young (ca. 1 Mya) re-colonisation of *C. densiflorum* from Central America to Amazonia is observed in our AAR. Likewise, only a biotical exchange can be stated between Central America and Choco despite their adjacency. Nevertheless, it remains unclear whether *Cycnoches* species from clade B have radiated from a common ancestor either distributed in the Choco region or Central America because of statistical uncertainty.

One of the most striking results of our study is that all migration and re-colonisation processes imply multiple dispersal events across the Andes. In late Miocene (i.e., the time when *Cycnoches* has started to diversify), Colombian and Venezuelan Northern Andes have already reached elevations up to 3000 m and more (Hoorn et al., 2010). Furthermore, intense migrations from Amazonia to Central America and back have taken place ~1 mya, when the Northern Andes already peaked around 4000 m elevations (see mean Northern Andean elevation in Fig. 3 – inset). Similar biogeographic patterns have been reported for bromeliads, in which several lineages such as Tillandsioidae and Hechtioideae have diversified from Guyana Shield and subsequently migrated across the Andes to Central

America around 15 mya (Givnish et al., 2011). Thus, efficiency of the Andes as barrier for reproductive isolation appears low for epiphyte, wind dispersed plant lineages.

The *Cycnoches egertonianum* species complex is composed of two clades showing a clear geographic separation (Fig. 2). Plants of the first clade (i.e., C. *amparoanum*, *C. egertonianum* var. *egertonianum, C. egertonianum* var. *viride* and *C. rossianum*) occur from southern Mexico, south-eastern Costa Rica and possibly north-eastern Panama, while those of the other clade (i.e., *C. densiflorum*, *C. guttulatum* and *C. pachydactylon*) are distributed from western to south-eastern Panama and northern Colombia. Surprisingly, the split between the two lineages has been dated to the late Pliocene, and their diversification may coincide with the formation of the Cordillera of Talamanca. The rise of this mountain range extending from the southern region of El Valle in Costa Rica to western Panama has taken place likewise during the Pliocene (6 to 3.4 mya) (Boer et al., 1995). Mountain building time of Cordillera of Talamanca has predated by almost 1.5 mya the diversification time of *Cycnoches'* Central American lineages nested in Clade C (Figure 3). Therefore, our results suggest that Northern Central American clades might have diverged from a common ancestor, which successfully crossed that range that reaches from 1800 to 3820 m.

Our biogeographical stochastic mapping reveals that within region speciation is among one of the most relevant phenomena (mean counts=16.34) identified for diversification of *Cycnoches*. The establishment of lineages in subtle but distinct micro-habitats in lowland wet forest has been invoked as one of the drivers of extremely high epiphyte diversity in the Amazonian region (Gentry & Dodson, 1987), and might have explanatory power for the high within-region diversity of *Cycnoches* as well. Niche accomplishment at particularly fine scale is assumed for epiphytes when they establish

populations in very specific, restricted microhabitats (e.g., understory, canopy, middle-story), which abound in lowland wet forests due to their large spatial heterogeneity (Baker, 1970). This is reflected in very high levels of endemism and abundance of vascular epiphytic species observed in relatively small lowland Amazonian forests patches. For example, Kreft et al. (2004) reported 8762 epiphytic individuals assigned to 146 different species on a 0.1 ha plot located at Tiputini (western Amazonas, Ecuador). Similar scenarios for within region speciation because of microhabitat specialisation have been also reported for other epiphytic orchid lineages such as the Neotropical *Telipogon* Kunth. Here, endemic species distributed in very small areas (e.g., "Nudo" de Pasto, Colombia, Northern Andes) compared with the entire geographic range of the lineage, are distributed each in very specific areas (slopes or valleys) (Gentry & Dodson, 1987).

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Biosketches

Oscar A. Pérez is a botanist interested on systematics, biogeography and evolution of Neotropical plant lineages. He is currently completing his PhD at the University of Munich, working on the molecular phylogenetics and biogeography of Darwin's most favourite orchids (subtribe Catasetinae).

Marc Gottschling is a Professor at the University of Munich working on development of evolutionary scenarios based on field work, taxonomy, morphology / anatomy and DNA-sequence comparison. His taxonomic focus is on the forget-me-not clade (Boraginales, flowering plants), unicellular dinophytes/-flagellates (protists) and papilloma viruses (particularly of non-human hosts). He is also interested in reliable application of scientific names and determining evolutionary mechanisms driving diversification of organisms.

Günter Gerlach is senior curator at the Botanical Garden Munich. His focus is on systematics, taxonomy, pollination and chemotaxonomy of Neotropical orchid subtribes pollinated by fragrance collecting male euglossine bees. Fragrance analyses of floral aroma from cultivated orchids mentioned form the base of his investigation. The attractiveness of fragrance substances found is tested in natural habitats to get information on the pollinators. Fragrance composition is also used to delimit the species because of high fragrance specificity in respective euglossine bees.

Author contributions

O.A.P.E. and M.G. designed research; O.A.P.E. and G.G. collected samples; O.A.P.E. performed all the lab work and analyses; all authors wrote the manuscript with the lead of O.A.P.E..

Tables

Table 1. Estimated node ages for selected divergence events lineages under a strict and relaxed molecular clock models. Bayesian posterior probabilities (BPP) for every node are provided. Maximum and minimum intervals correspond to 95% posterior probability interval values.

	Strict clock				Relaxed clock			
node	**Age**	**Minimum interval**	**Maximum interval**	**BPP**	**Age**	**Minimum interval**	**Maximum interval**	**BPP**
Root (A)	31.65	19.28	45.39	1	27.87	15.46	53.77	1
LRCA Catasetinae (B)	21.76	15.24	28.39	1	21.73	14.79	28	1
C	17.62	11.9	26.3	1	17.94	11.05	26.09	1
Catasetum (D)	1.87	0.76	4.27	1	1.87	0.56	6.2	1
E	14.68	9.73	21.11	1	14.64	8.19	23.43	1
Mormodes + *Cycnoches* (F)	12.21	8.28	17.68	1	11.81	6.44	18.98	1
LRCA *Cycnoches* (G)	5.95	3.59	9.2	1	6.15	3.18	12.21	1
LRCA Clade B + C (H)	3.74	2.22	6.14	1	3.93	1.98	7.82	1
Clade B (I)	1.75	0.89	3.54	1	2.29	0.9	5.56	1
Clade C (J)	2.17	1.07	3.9	1	1.82	0.8	5.46	1
Mormodes (K)	5.04	3	8.01	1	5.24	2.34	11.66	1

Table 2. Comparison of different models as implemented in DEC, DIVA and BAYAREALIKE. Akaike Information Criterion (AIC) results, including model weights and the corresponding ratios are provided.

		Parameter estimates				Likelihood Ratio Test				AIC analysis				
Model	**Ln *L***	**Number**	***d***	***e***	***j***	**Alt Ln *L***	**null Ln *L***	***D***	***P-value***	**AIC 1**	**AIC 2**	**wt 1**	**wt2**	**Ratio**
DEC	-50.2386	2	0.0231544	0.014804	-	-33.414	-50.2386	33.65	6.60E-09	72.83	104.5	1	1.30E-07	7453181
DEC+J	-33.4145	3	1.00E-12	1.00E-12	0.07636									
DIVALIKE	-51.0356	2	0.0298684	0.015309	-	-32.7180	-51.0356	36.64	1.40E-09	71.44	106.1	1	3.00E-08	3.32E+07
DIVALIKE+J	-32.7180	3	1.00E-12	1.00E-12	0.07303									
BAYAREALIKE	-72.3620	2	0.048978	0.105589	-	-34.3464	-72.362	76.03	2.80E-18	74.69	148.7	1	8.40E-17	1.19E+16
BAYAREALIKE+J	-34.3464	3	1.00E-07	1.00E-07	0.0773									

d: Dispersal; e: Extinction; j: Founder

Table 3. Biogeographical stochastic mapping event counts in 50 iterations. Mean and standard deviation for every event are provided.

	DIVA		DIVA+J	
Parameter	**mean**	**SD**	**mean**	**SD**
d (dispersal)	6.74	0.78	0	0
e (extinction)	0	0	0	0
a (range switching)	0	0	0	0
y (sympatry)	16.16	0.91	16.34	0.89
s (subset sympatry)	0	0	0	0
v (vicariance)	6.84	0.91	0.04	0.2
j (founder)	0	0	6.62	0.85

Figures

Figure 1. Main morphological traits of *Cycnoches*. Habit of *C. peruvianum* Rolfe (A), *C. rossianum* (B) and *C. egertonianum* (C). Homoblastic pseudobulbs are pointed with an arrow. Functionally staminate (D) and pistillate (E) flowers of *C. ventricosum*, a member of sect. *Cycnoches*. Functionally staminate (F) and pistillate G) flowers of *C. herrenhusanum*, a member of sect. *Heteranthae*. Note the difference between the elongated column of the staminate flowers (pointed with an arrow in D and F) and the, short, stout column in the pistillate flowers (pointed with an arrow in E and G).

Figure 2. Best scoring, ML tree of *Cycnoches* obtained from non-conflicting concatenated nuclear ETS, ITS, *Xdh* and chloroplast *trn*S-G, *ycf*1 loci. Node charts indicate Likelihood Bootstrap Support (LBS > 75), in where fully red diagrams indicate LBS 100. Numbers at nodes indicate Bayesian Posterior Probability (BPP > .95). Representatives of each clade are shown in pictures. For clade A, *Cycnoches haagii*; for clade B, *C. chlorochilon* Klotzsch; for clade C, *C. herrenhusanum* (up), *C. peruvianum* (middle) and *C. guttulatum* (bottom).

Figure 3. Chronogram for *Cycnoches* obtained under a relaxed clock model, applied to a non-conflicting, concatenated nuclear (ITS, ETS, *Xdh*) and chloroplast (*trn*S-G, *ycf1*) loci. Node bars indicate 95% posterior probability intervals. Numbers at nodes indicate Bayesian Posterior Probability (BPP > .95). Age estimations, including maximum and minimum intervals for labeled nodes, are provided in Table 1. Calibration points (LCA of Catasetinae and tree root) is highlighted with a black circle. Time scale is provided in million years (mya). Node charts correspond to ancestral areas estimated under the dispersal-vicariance model, including founder event process (*J*). Blue arrows indicate estimated times of some major mountain building processes in Northern Andes. Pink and green lines indicate mean

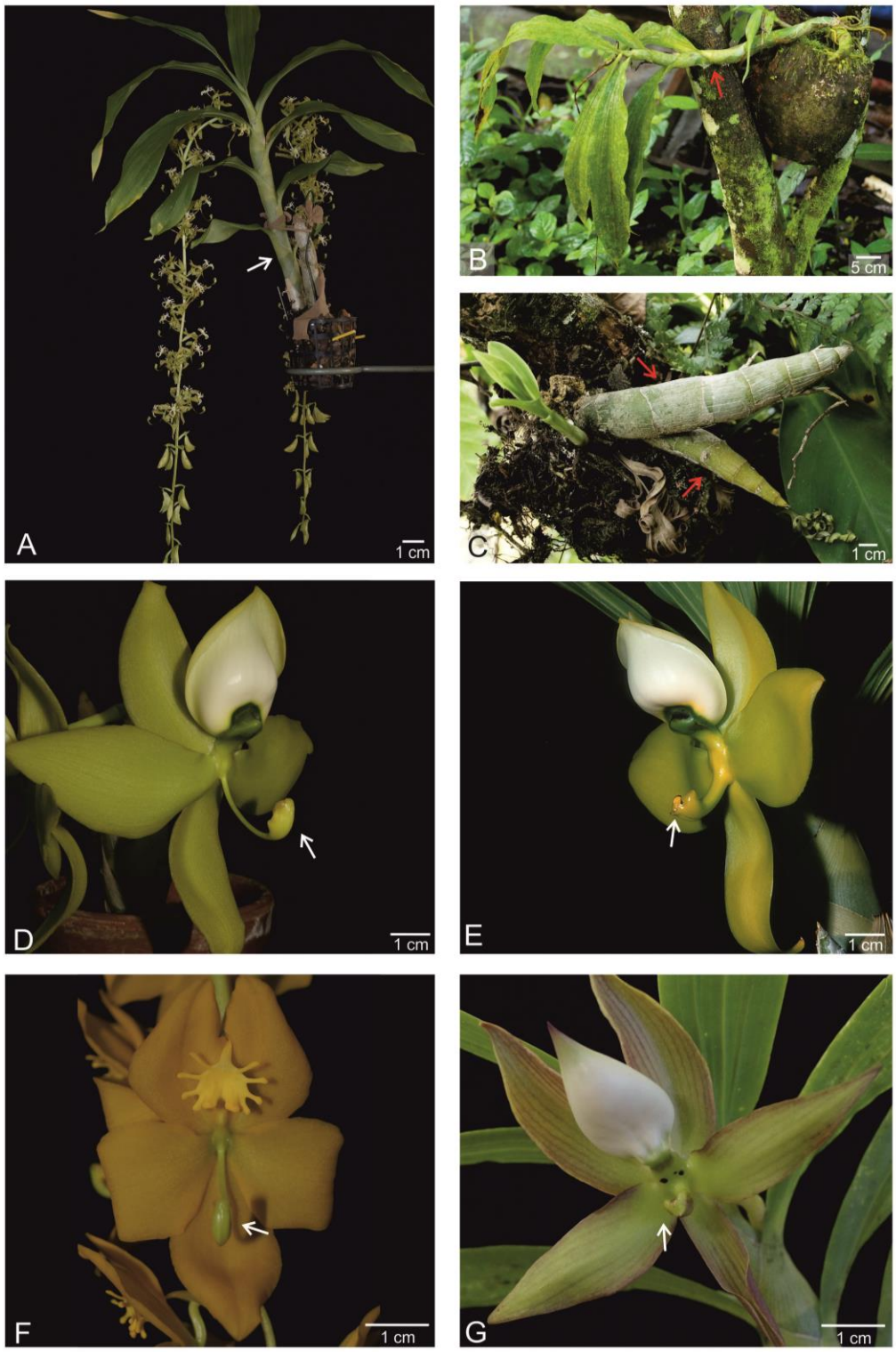
elevations (m) on Colombian and Venezuelan Northern Andes, respectively (adapted from Hoorn et al 2010). Members of the *Cycnoches egertonianum* species complex are highlighted in bold. (Inset) Coded areas used for biogeographic analysis are listed as follows: Central America (blue); Choco (green); Amazonas (yellow). Political divisions and elevation data from DIVA-GIS (http://www.diva-gis.org/gdata)

Supporting Information

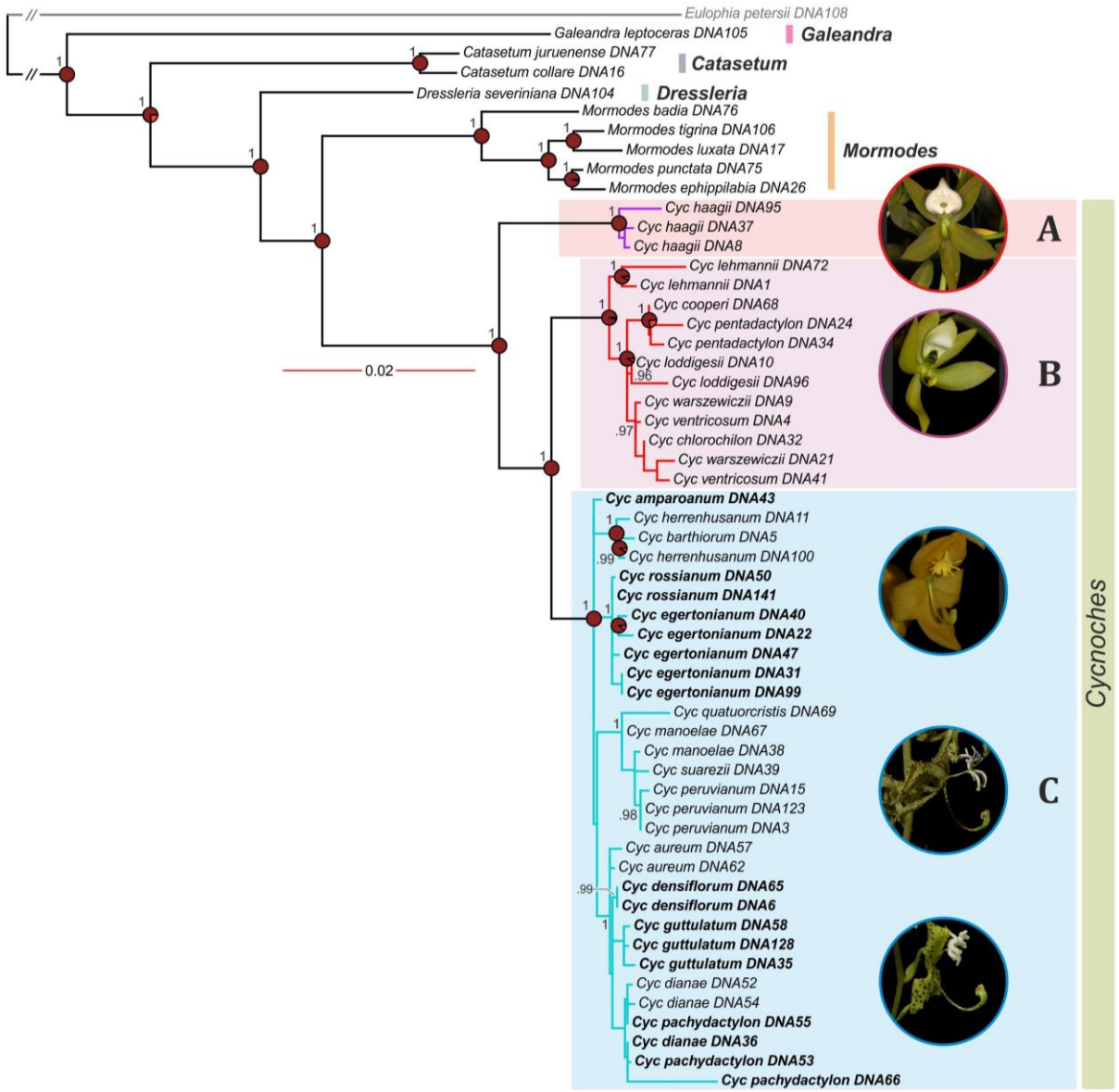
Additional Supporting Information may be found in the online version of this article:

Appendix S1. Individual **n**uclear and chloroplast derived phylogenies, results of PACo analysis, species distribution map, voucher list, primers, alignment characterization and strict and relaxed molecular clock models derived chronograms.

Figure 1.



727 **Figure 2.**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Galeandra
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Catasetum collare DNA16
Catasetum
Dressleria severiniana DNA104
Dressleria
Mormodes badia DNA76
Mormodes tigrina DNA106
Mormodes luxata DNA17
Mormodes punctata DNA75
Mormodes ephippilabia DNA26
Mormodes
Cyc haagii DNA95
Cyc haagii DNA37
Cyc haagii DNA8
A
Cyc lehmannii DNA72
Cyc lehmannii DNA1
Cyc cooperi DNA68
Cyc pentadactylon DNA24
Cyc pentadactylon DNA34
Cyc loddigesii DNA10
Cyc loddigesii DNA96
Cyc warszewiczii DNA9
Cyc ventricosum DNA4
Cyc chlorochilon DNA32
Cyc warszewiczii DNA21
Cyc ventricosum DNA41
B
0.02
Cyc amparoanum DNA43
Cyc herrenhusanum DNA11
Cyc barthiorum DNA5
Cyc herrenhusanum DNA100
Cyc rossianum DNA50
Cyc rossianum DNA141
Cyc egertonianum DNA40
Cyc egertonianum DNA22
Cyc egertonianum DNA47
Cyc egertonianum DNA31
Cyc egertonianum DNA99
Cyc quatuorcristis DNA69
Cyc manoelae DNA67
Cyc manoelae DNA38
Cyc suarezii DNA39
Cyc peruvianum DNA15
Cyc peruvianum DNA123
Cyc peruvianum DNA3
C
Cyc aureum DNA57
Cyc aureum DNA62
Cyc densiflorum DNA65
Cyc densiflorum DNA6
Cyc guttulatum DNA58
Cyc guttulatum DNA128
Cyc guttulatum DNA35
Cyc dianae DNA52
Cyc dianae DNA54
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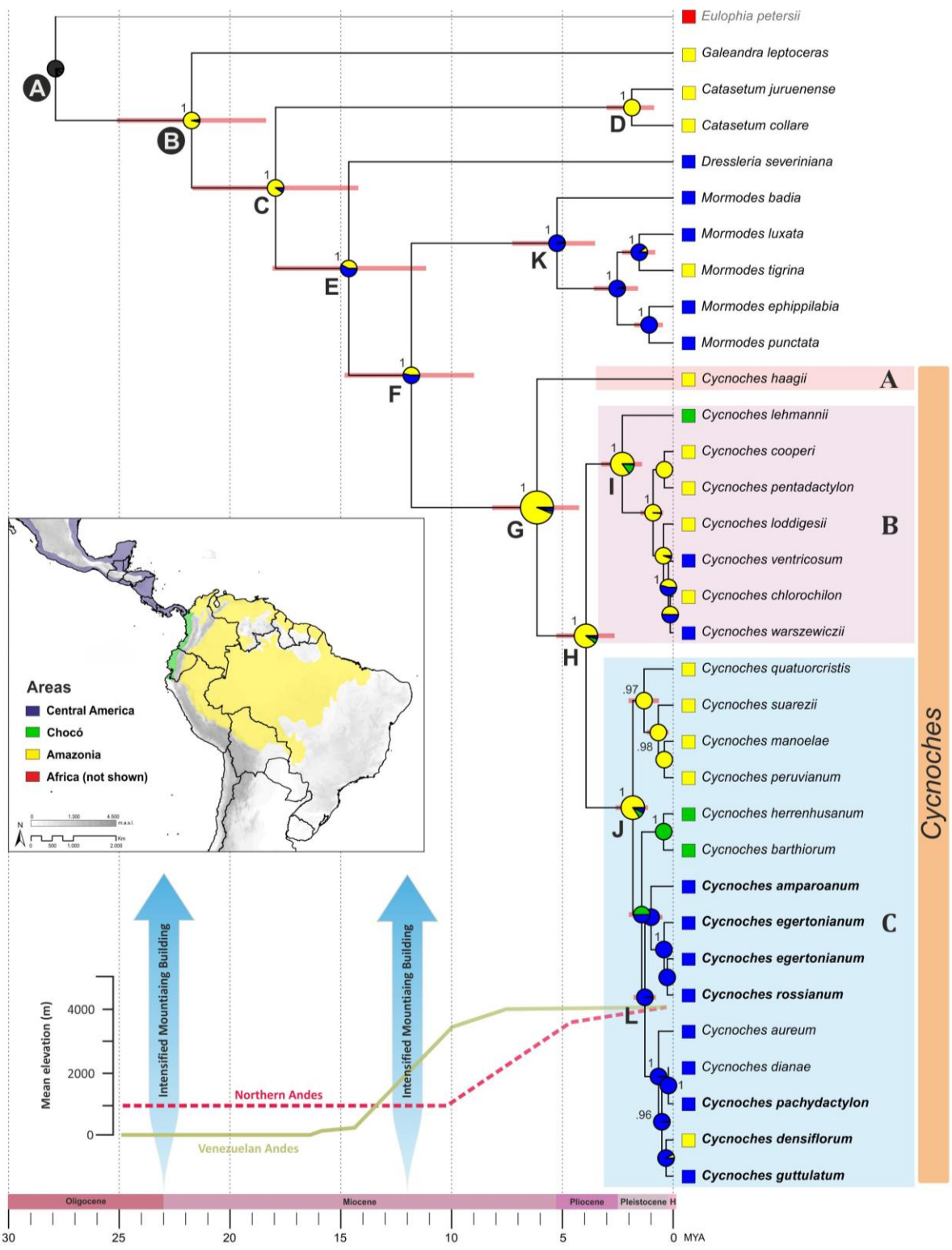
Cyc dianae DNA36
Cyc pachydactylon DNA53
Cyc pachydactylon DNA66
Cycnoches

Figure 3.


Eulophia petersii
Galeandra leptoceras
Catasetum juruenense
Catasetum collare
Dressleria severiniana
Mormodes badia
Mormodes luxata
Mormodes tigrina
Mormodes ephippilabia
Mormodes punctata
Cycnoches haagii
Cycnoches lehmannii
Cycnoches cooperi
Cycnoches pentadactylon
Cycnoches loddigesii
Cycnoches ventricosum
Cycnoches chlorochilon
Cycnoches warszewiczii
Cycnoches quatuorcristis
Cycnoches suarezii
Cycnoches manoelae
Cycnoches peruvianum
Cycnoches herrenhusanum
Cycnoches barthiorum
Cycnoches amparoanum
Cycnoches egertonianum
Cycnoches egertonianum
Cycnoches rossianum
Cycnoches aureum
Cycnoches dianae
Cycnoches pachydactylon
Cycnoches densiflorum
Cycnoches guttulatum
A
B
C
Cycnoches
Areas
Central America
Chocó
Amazonia
Africa (not shown)
Mean elevation (m)
4000
2000
0
Intensified Mountiaing Building
Intensified Mountiaing Building
Northern Andes
Venezuelan Andes
Oligocene
Miocene
Pliocene
Pleistocene
30
25
20
15
10
5
0 MYA

HISTORICAL BIOGEOGRAPHY OF *CYCNOCHES* (CATASETINAE): THE IMPROBABLE JOURNEYS OF SWAN ORCHIDS ACROSS THE ANDES

Oscar Alejandro Pérez-Escobar; Marc Gottschling; Günter Gerlach

Supporting Information – Appendix S1

Including:

Figure S1-S5

Tables S1-S3

Figure S1. Conflicting nuclear – chloroplast associations (i.e. pair of nuclear / chloroplast taxa) obtained by PACo analysis, using posterior probability trees. Taxa with normalized squared residual values above the cut-off value (red line; see species names highlighted in red) indicate potential conflicting associations.

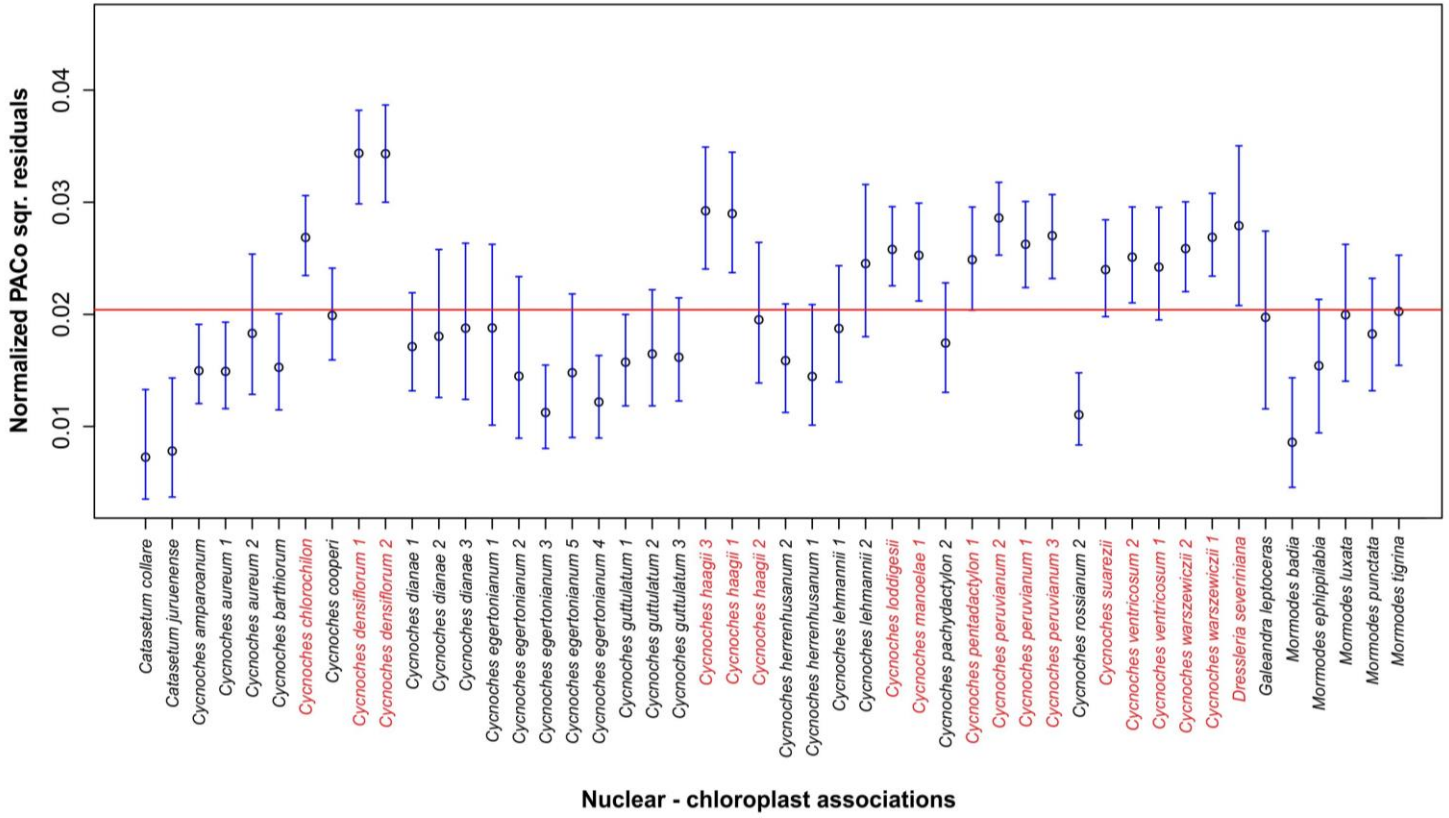


Figure S2. Distribution map of *Cycnoches* based on herbarium and field records.

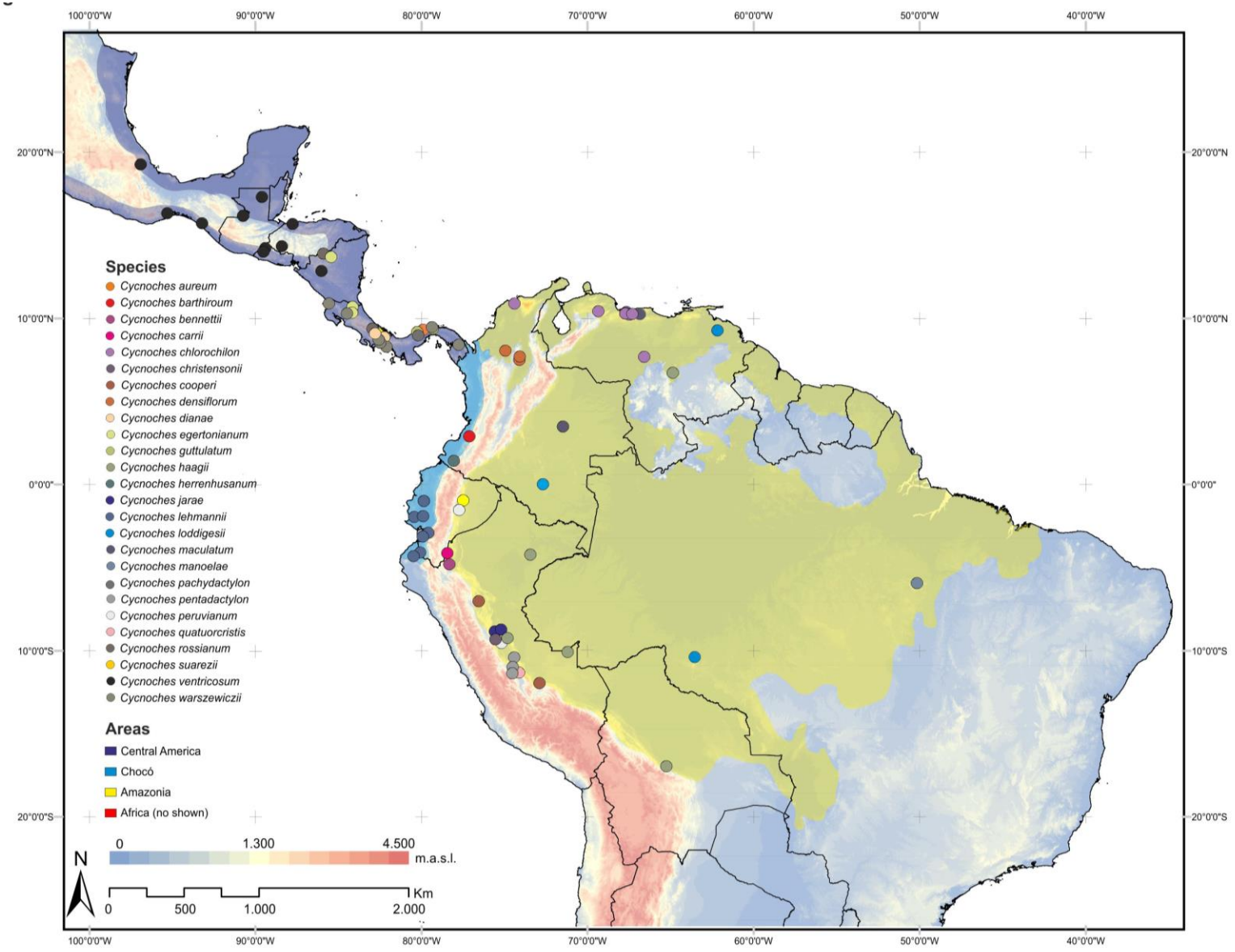


Figure S3. Phylogenetic relationships of *Cycnoches* independently derived from nuclear (ITS, ETS, *Xdh*) and chloroplast loci (*trn*S-G, *ycf1*) datasets. Node charts indicate Likelihood Bootstrap Support (LBS > 75), in where fully red diagrams indicate LBS 100. Numbers on node indicate Bayesian Posterior Probability (BPP > .95). Clades recovered in the nuclear phylogeny are color coded onto the chloroplast phylogeny.

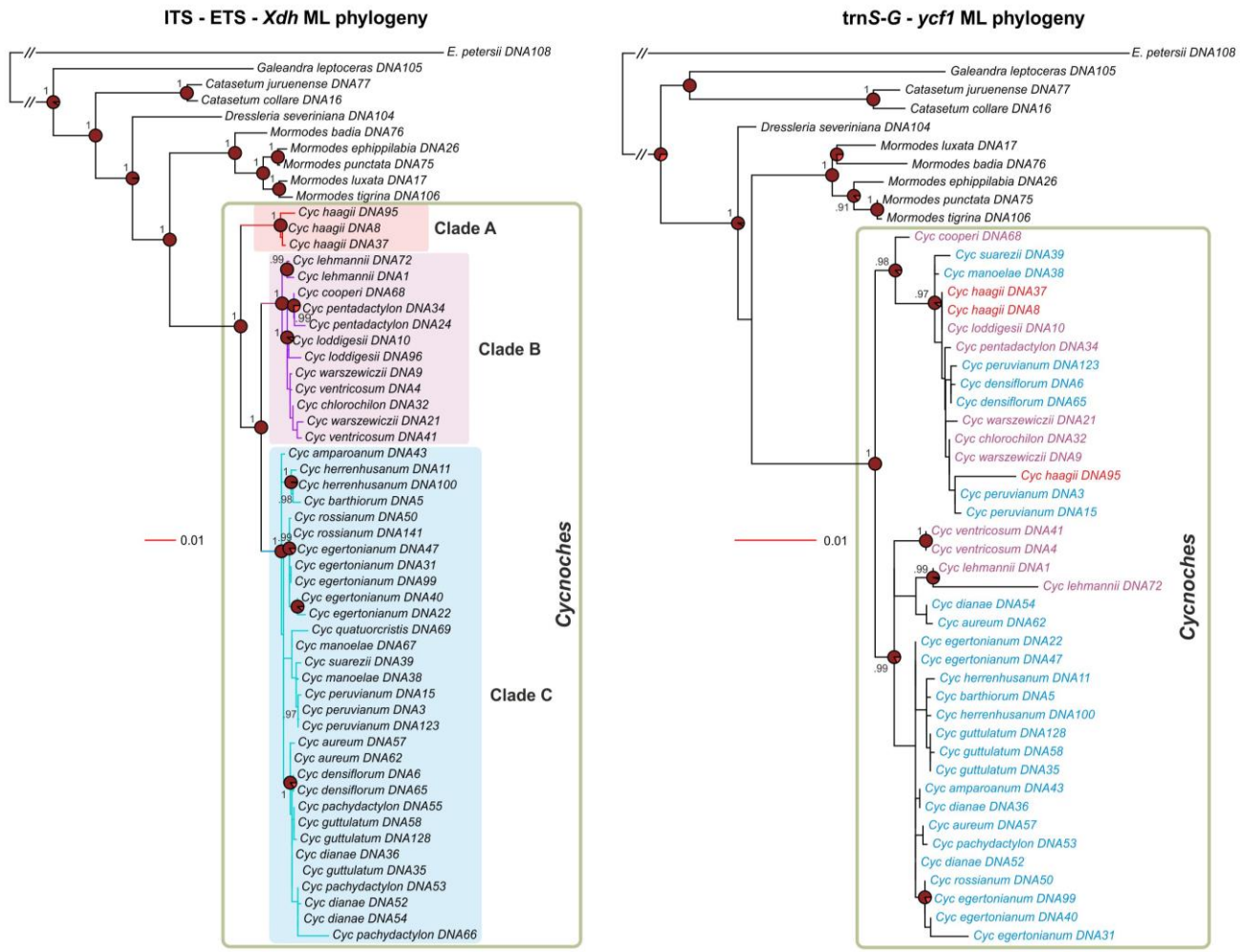


Figure S4. Chronogram for *Cycnoches* obtained under a strict clock model, applied to a non-conflicting, concatenated nuclear (ITS, ETS, *Xdh*) and chloroplast (*trn*S-G, *ycf1*) loci. Node bars indicate 95% posterior probability intervals. Age estimations, including maximum and minimum intervals for labeled nodes, are provided in Table 1.

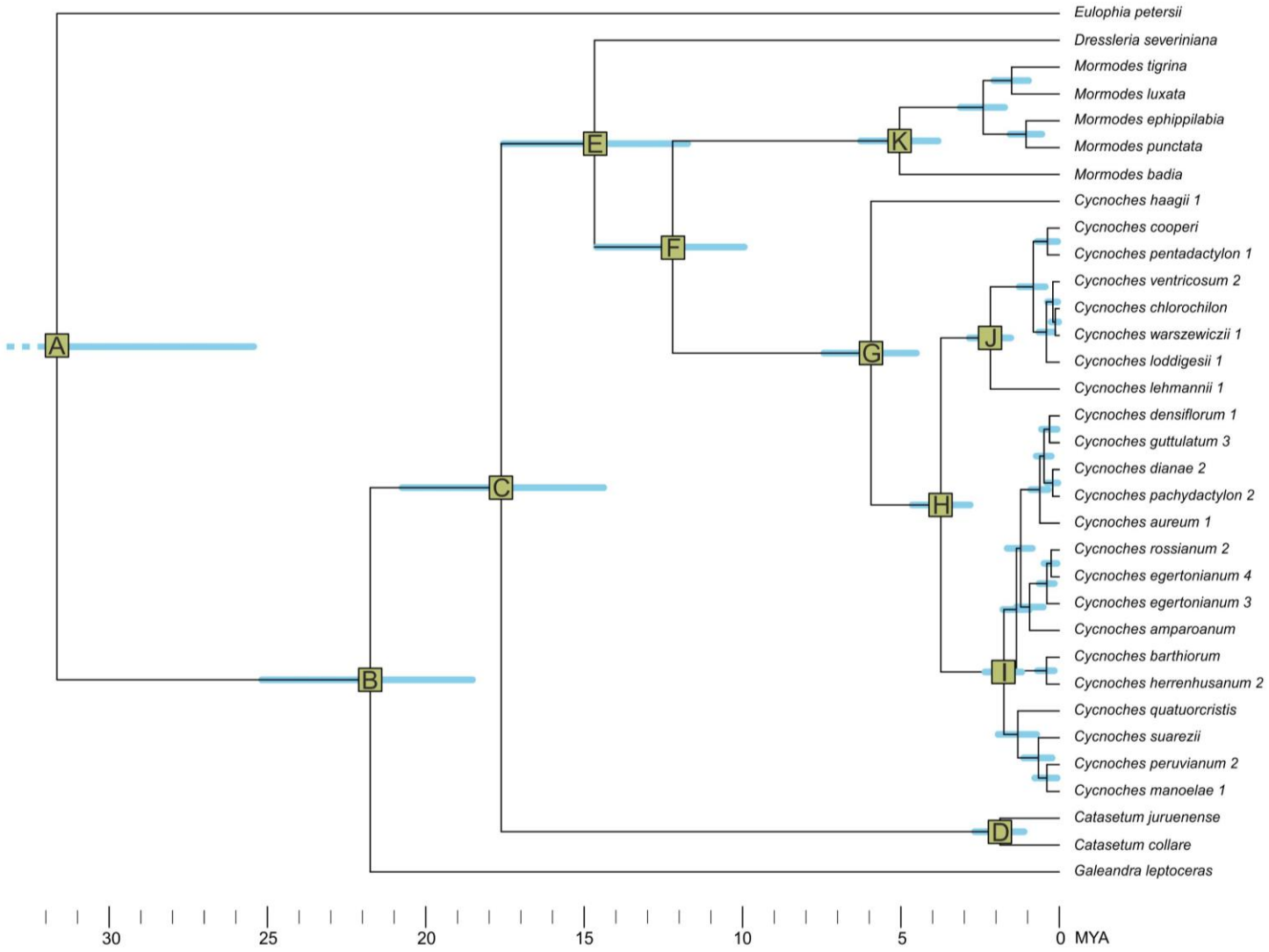


Figure S5. Chronogram for *Cycnoches* obtained under a relaxed clock model, applied to a non-conflicting, concatenated nuclear (ITS, ETS, *Xdh*) and chloroplast (*trn*S-G, *ycf1*) loci. Node bars indicate 95% posterior probability intervals. Age estimations, including maximum and minimum intervals for labeled nodes, are provided in Table 1.

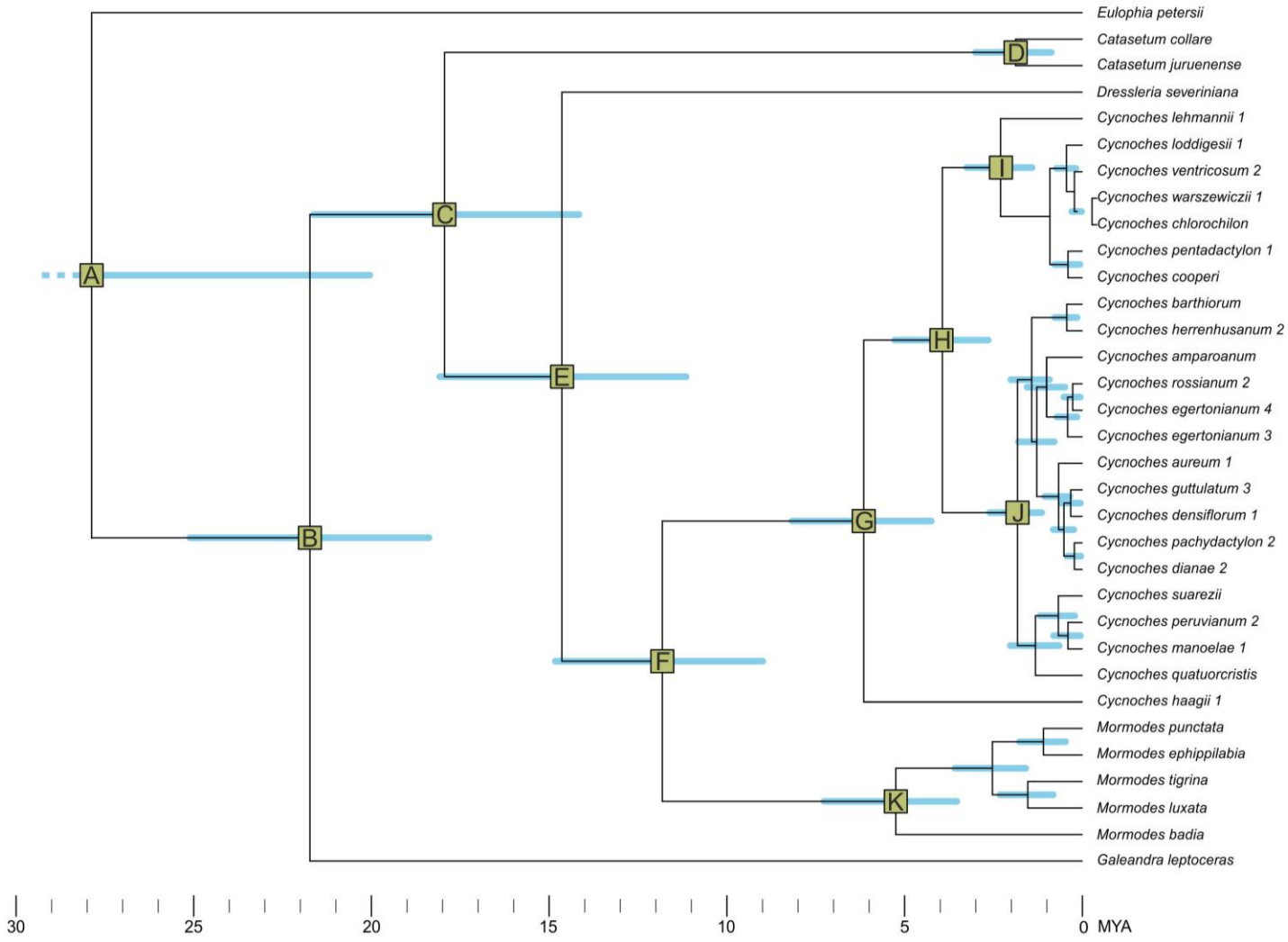


Table S1. Species names and voucher information for material used in this study. Taxa sequenced in this study are indicated in bold.

Species	Voucher	Distribution	ITS	ETS	*Xdh*	*Trn*S-G	*ycf1*
Cycnoches amparoanum Schltr.	Perez 1413 (M)	Costa Rica	#GBN	#GBN	#GBN	#GBN	#GBN
Cycnoches aureum Lindl. & Paxton	(1) Perez & Gerlach 1473 (M)	Panama	#GBN	#GBN	#GBN	#GBN	#GBN
	(2) Perez & Gerlach 1480 (M)		#GBN	#GBN	-	-	#GBN
Cycnoches barthiorum G.F.Carr & Christenson	BGM 12/1476 (M)	Colombia	#GBN	#GBN	#GBN	#GBN	#GBN
Cycnoches chlorochilon Klotzsch	BGM 94/0981 (M)	Colombia, Venezuela	#GBN	#GBN	#GBN	#GBN	#GBN
Cycnoches cooperi Rolfe	Whitten W-3591 (FLAS)	Peru	#GBN	#GBN	#GBN	#GBN	-
Cycnoches densiflorum Rolfe	(1) BGH Kusibab 5/2004 (M)	Colombia, Panama	#GBN	#GBN	#GBN	#GBN	#GBN
	(2) Perez 1486 (M)		#GBN	#GBN	-	-	#GBN
Cycnoches dianae Rchb. f.	(1) BGM 12/0841 (M)	Panama	#GBN	#GBN	-	#GBN	#GBN
	(2) Perez & Gerlach 1468 (M)		#GBN	#GBN	#GBN	-	#GBN
	(3) Perez & Gerlach 1470 (M)		#GBN	#GBN	#GBN	-	#GBN
Cycnoches egertonianum Bateman	(1) Whitten 3821 (FLAS)	S. Mexico to N. Costa Rica	#GBN	#GBN	-	-	#GBN
	(2) BGM 12/1471 (M)		#GBN	#GBN	#GBN	#GBN	-
	(3) Franke sn (MEXU)		#GBN	#GBN	#GBN	#GBN	#GBN
	(4) BGM 13/2483 (M)		#GBN	#GBN	#GBN	#GBN	#GBN
	(5) Perez 1463 (M)		#GBN	#GBN	-	-	#GBN
Cycnoches guttulatum Schltr.	(1) BGM 13/2505 (M)	Panama	#GBN	#GBN	#GBN	-	-
	(2) BGM 12/2124 (M)		#GBN	#GBN	-	-	#GBN
	(3) Perez & Gerlach 1476 (M)		#GBN	#GBN	#GBN	#GBN	#GBN
Cycnoches haagii Barb. Rodr.	(1) BGH Brock 10/72 (M)	Brazil, Bolivia, Peru, Venezuela	#GBN	#GBN	#GBN	#GBN	#GBN
	(2) BGM 05/1232 (M)		#GBN	#GBN	-	-	#GBN
	(3) BGM 12/0843 (M)		#GBN	#GBN	#GBN	#GBN	#GBN
Cycnoches herrenhusanum Jenny & G.A. Romero	(1) BGH Hubein 1/78 (M)	Colombia	#GBN	-	-	#GBN	#GBN
	(2) BGM 12/0871 (M)		#GBN	#GBN	#GBN	#GBN	#GBN
Cycnoches lehmannii Rchb. f.	(1) BGH T1/97 (M)	Ecuador	#GBN	#GBN	#GBN	#GBN	#GBN
	(2) Whitten ABG 1989-342 (FLAS)		#GBN	#GBN	#GBN	-	#GBN
Cycnoches loddigesii Lindl.	(1) BGH H9/70 (M)	French Guiana, Suriname, Venezuela, Brazil	#GBN	#GBN	-	#GBN	#GBN
	(2) BGM 93/3573 (M)		#GBN	#GBN	#GBN	-	#GBN

Cycnoches manoelae V.P. Castro & Campacci	(1) BGM 12/2255 (M)	Brazil	#GBN	#GBN	#GBN	#GBN	#GBN
	(2) Gerlach 05/1231 (FLAS)		#GBN	#GBN	#GBN	-	-
Cycnoches pachydactylon Schltr.	(1) Gerlach 00/3415 (FLAS)	Panama	#GBN	-	-	-	-
	(2) Perez & Gerlach 1469 (M)		#GBN	#GBN	#GBN	#GBN	#GBN
	(3) Perez & Gerlach 1471 (M)		#GBN	-	-	-	-
Cycnoches pentadactylon Lindl.	(1) BGM 13/1195 (M)	Brazil, Peru	#GBN	#GBN	-	-	#GBN
	(2) H. Hills F1814 (FLAS)		#GBN	#GBN	-	-	-
Cycnoches peruvianum Rolfe	(1) BGH Kusibab 5/04 (M)	Ecuador, Peru	#GBN	-	#GBN	#GBN	#GBN
	(2) BGM X/1351 (M)		#GBN	#GBN	#GBN	#GBN	#GBN
	(3) Perez 1402 (M)		#GBN	#GBN	#GBN	-	#GBN
Cycnoches quatuorcristis D.E.Benn.	Whitten 3834 (FLAS)	Peru	#GBN	#GBN	-	-	-
Cycnoches rossianum Rolfe	(1) Gomez & Perez 1496 (M)	S. Mexico to N. Costa Rica	#GBN	-	-	-	-
	(2) BGM 14/1832 (M)		#GBN	#GBN	#GBN	#GBN	#GBN
Cycnoches suarezii Dodson	BGM 12/0836 (M)	Ecuador	#GBN	#GBN	#GBN	#GBN	#GBN
Cycnoches ventricosum Bateman	(1) Franke sn (MEXU)	S. Mexico to N. Nicaragua	#GBN	#GBN	-	#GBN	#GBN
	(2) Perez 1401 (M)		#GBN	#GBN	#GBN	-	#GBN
Cycnoches warszewiczii Rchb. f.	(1) BGH H1/73 (M)	S.Nicaragua to Panama	#GBN	#GBN	#GBN	#GBN	#GBN
	(2) BGH Horich 12/75 (M)		#GBN	#GBN	#GBN	#GBN	#GBN
OUTGROUP							
Catasetum collare Cogn.	BGM 05/1000 (M)	Brazil, Venezuela	#GBN	#GBN	#GBN	-	#GBN
Catasetum juruenense Hoehne	BGM 05/1223 (M)	Brazil	#GBN	#GBN	#GBN	-	#GBN
Dressleria severiniana H.G.Hills	BGM 14/1196 (M)	Panama	#GBN	-	#GBN	-	#GBN
Eulophia petersii (Rchb.f.) Rchb.f.	BGM 11/3891 (M)	Tropical Africa	#GBN	-	-	#GBN	#GBN
Galeandra leptoceras Schltr.	BGM 12/2403 (M)	Colombia	#GBN	#GBN	#GBN	#GBN	#GBN
Mormodes badia Rolfe ex W.Watson	BGM 02/2840 (M)	Mexico	#GBN	#GBN	#GBN	#GBN	#GBN
Mormodes ephippilabia Fowlie	BGM 03/0775 (M)	Honduras, Costa Rica	#GBN	#GBN	#GBN	-	#GBN
Mormodes luxata Lindl.	BGM 92/3103 (M)	Mexico	#GBN	#GBN	#GBN	-	#GBN
Mormodes punctata Rolfe	Perez & Gerlach 1483 (M)	Panama	#GBN	#GBN	#GBN	#GBN	#GBN
Mormodes tigrina Barb. Rodr.	BGM 03/0773 (M)	Brazil	#GBN	#GBN	#GBN	#GBN	#GBN
Mormodes tigrina Barb. Rodr.	BGM 03/773	Brazil	#GBN	#GBN	#GBN	#GBN	#GBN

Table S2. Primer and PCR settings used for amplifying chloroplast and nuclear DNA loci.

Loci	Primer	Sequence	Reference	Pre-melt	Amplification	Final extension	Number of amplification cycles
ITS	ITS 4	TCC-TCC-GCT-TAT-TGA-TAT-GC	Baldwin (1992)	95°C (3 min)	95°C (30 secs) + 52°C (1 min) + 68°C (1 min)	68°C (10 min)	39
	ITS 5	GGA-AGT-AAA-AGT-CGT-AAC-AAG-G		95°C (3 min)	95°C (30 secs) + 52°C (1 min) + 68°C (1 min)	68°C (10 min)	39
ETS	EST-Orchid	CAT-ATG-AGT-TGT-TGC-GGA-CC (AT)-T	Monteiro et al. (2010)	95°C (3 min)	95°C (30 secs) + 52°C (1 min) + 68°C (1 min)	68°C (10 min)	39
	18-IGS	AGA-CAA-GCA-TAT-GAC-TAC-TGG-CAG-G	Balwin and Markos (1998)	95°C (3 min)	95°C (30 secs) + 52°C (1 min) + 68°C (1 min)	68°C (10 min)	39
Xdh	X502F	TGT-GAT-GTC-GAT-GTA-TGC	Górniak et al. (2010)	95°C (3 min)	95°C (30 secs) + 53°C (1 min) + 68°C (1.5 min)	68°C (10 min)	39
	X1599R	G(AT)G-AGA-GAA-A(CT)TG-GAG-CAA-C		95°C (3 min)	95°C (30 secs) + 53°C (1 min) + 68°C (1.5 min)	68°C (10 min)	39
Ycf1	3720F	TAC-GTA-TGT-AAT-GAA-CGA-ATG-G	Neubig et al. (2009)	95°C (3 min)	95°C (30 secs) + 54°C (1 min) + 68°C (1.5 min)	68°C (10 min)	39
	5500R	GCT-GTT-ATT-GGC-ATC-AAA-CCA-ATA-GCG		95°C (3 min)	95°C (30 secs) + 54°C (1 min) + 68°C (1.5 min)	68°C (10 min)	39
*trn*S-G	trn-S(GCU)	GCC-GCT-TTA-GTC-CAC-TCA-GC	Hamilton (1999)	95°C (3 min)	95°C (30 secs) + 51.5°C (1 min) + 68°C (1.5 min)	68°C (10 min)	39
	trn-G(UCC)	GAA-CGA-ATC-ACA-CTT-TTA-CCA-C		95°C (3 min)	95°C (30 secs) + 51.5°C (1 min) + 68°C (1.5 min)	68°C (10 min)	39

Table S3. Alignment characterisation.

	ETS	**ITS**	***Xdh***	***trn*S-G**	***ycf1***
Number of cells	49/56	56/56	39/56	31/56	47/56
Alignment length (bp)	544	848	1004	792	1642
Parsimony Informative Sites (no/%)	123/23%	140/16%	49/5%	80/10%	114/7%

Chapter 8

General Discussion

Phylogenetic relationships within Catasetinae and *Cycnoches*

Internal phylogenetic relationships of Catasetinae were poorly understood because of the lack of a comprehensively sampled dataset. Likewise, previous molecular phylogenetic studies of *Cycnoches* included no more than three species in their sampling (e.g. Pridgeon & Chase, 1998; Batista et al., 2014; Whitten et al., 2014), thus keeping elusive their internal relationships. The molecular phylogeny of Catasetinae (**Chapter 5 and 6**) I produced during my research is the first to include representative taxa from all accepted eight lineages, sampled from nuclear and chloroplast loci. In addition, the molecular phylogeny of *Cycnoches* stands as the first effort to investigate the internal phylogenetic relationships of this lineage and as the most comprehensive phylogeny produced to date by including nuclear and chloroplast sequences of 23 of the 34 known extant species. Therefore, these studies have significantly contributed to the knowledge of the Orchidaceae by enlightening previously obscured phylogenetic relationships and providing a solid foundation for further studies about evolution of sexual systems (**Chapter 6**), pollination syndromes and historical biogeography **(Chapter 7**).

In Catasetinae, the inclusion of samples from previously unavailable lineages (e.g. *Catasetum, Mormodes* and *Cycnoches*), results in a representatively sampled phylogeny with maximal statistical support (Bayesian posterior probability and Maximum Likelihood bootstrap support) for all generic lineages and provides high statistical support for almost all nodes across the phylogeny. I aimed at including as many representatives as possible from each generic lineage of Catasetinae, and in the particular case of *Cycnoches*, all extant taxa. To achieve such comprehensive sampling, I did intensive field work in many Latin American countries, namely Colombia, Costa Rica, Mexico, Nicaragua and Panama, but also gathered material from herbarium specimen loans from five major herbaria. Nevertheless, it was a very challenging task to get samples from representative species of certain biogeographical regions. This is because *i*) often orchid species have very narrow distribution ranges (Cribb et al., 2003; Dodson, 2003); *ii*), individuals are very scarce due habitat loss and selective extraction by orchid smugglers (Neng, 2010) and iii) orchid material is scarce in herbarium collections as well for the reasons before mentioned. Moreover, once the samples were obtained, I encountered collection permit and export documentation issues to legally access and use the material for research purposes. For this reason, I could not rely on material obtained in Colombia and Nicaragua to increase my sampling. These conditions

have hindered primary research on several Latin American countries for many years already, and have been often encountered by many researchers working with Neotropical biodiversity, especially with endangered flora and fauna (Mulliken, 2009).

The utility of co-phylogenetic tools in the quantification of phylogenetic incongruence and their potential biological causes in Catasetinae

Incongruent phylogenetic relationships between nuclear and organelle DNA sequences are commonly found across several angiosperm lineages (Rieseberg et al., 1990; Fehrer et al., 2007, 2009; Salichos et al., 2014). More importantly, they often represent a huge challenge for researchers because they "undermine" reconstruction of evolutionary relationships (Rokas et al., 2003). In my research, the Catasetinae phylogeny derived from nuclear ETS, ITS, and *Xdh* DNA sequences reveals several highly supported conflicting phylogenetic positions when compared with the corresponding chloroplast *trn*L-F and *ycf*1 tree (Fig. 3 of **Chapter 5**). Several features of Catasetinae orchids such as reported natural hybrids (i.e. potential for hybridization: Romero-González & Carnevali, 1990, 1991, 1992; Romero & Jenny, 2009) and the occurrence of phylogenetic conflicts provide a unique opportunity to study in detail the utility of alternative approaches to address the discordance and detect putative conflicting associations between phylogenies. In addition, it offers a suitable opportunity to address a largely overlooked aspect of comparative phylogenetic methods (e.g. PACo and ParaFit), namely the efficiency of these approaches under different input data settings (i.e. phylograms and cladograms) (de Vienne et al., 2011; Cusimano & Renner, 2014).

The output of extensive simulation approaches and analysis of Catasetinae nuclear and chloroplast datasets illustrates the higher reliability of PACo compared with ParaFit in retrieving potential outlier associations (Fig. 4 of **Chapter 5**). It reveals that the performance of this approach is inversely proportional to the proportion of conflicting terminals included in the analysed datasets. ParaFit in contrast, do not successfully retrieves potential outliers, either with small or large proportion of conflicting OUTs included in the phylogenies (Supplementary Fig. S7 of **Chapter 5**), a result that was recovered by Balbuena et al., (2013) as well. Therefore, the number of correctly retrieved potential outlier decreases in PACo when the number of conflicting

OTUs is high in relation to the total number of OTUs included in the datasets. More importantly, the *in-silico* simulations indicate an underestimation of the number of potential conflicting outliers retrieved by PACo and ParaFit when cladograms are used over phylograms as input data. This is of particular importance because such tools and other distance-based comparative phylogenetic methods are often used, generally without indicating the kind of input data employed (de Vienne et al., 2011). Nevertheless, the branch length impact of input trees on the performance of these tools has been elusive. Even though the efficiency of PACo and Parafit is overall poor with cladograms, the use of phylograms or cladograms as input represents for the end-user a trade-off between accounting for evolutionary distances from the taxa analysed or for the pure topology only. By employing phylograms as input data, relative rates of evolution are considered at the cost of producing artefacts by the attraction in the distance matrix of unrelated taxa with short branches and departure of those with longer branches (de Vienne et al., 2012). This problem however might be tackled by including in the analysis comparisons with cladograms, which will consider only consensus topologies but not evolutionary rates. Hence, for a more sensitive analysis, the results of my study encourage the use of both phylograms and cladograms.

Several biological phenomena (e.g. ILS, HGT) are responsible for discordances between phylogenies, but all of them are very hard to identify in phylogenies when a few set of gene trees are available only (van der Niet & Linder, 2008). Phylogenetic incongruence in Catasetinae might be derived from chloroplast capture via past hybridization events. Chloroplast capture, the result of the introgression of a chloroplast genome from a foreign plant species into another, has been invoked as an explanation for topological incongruence between nuclear and chloroplast phylogenies (Tsitrone et al., 2003; Okuyama et al., 2005; Renoult et al., 2009; Nauheimer et al., 2012; Stegemann et al., 2012). Tsitrone et al (2003) provides a theoretical model to demonstrate that conditions (in lineages with chloroplast maternal inheritance) such as partial or complete cytoplasmic male sterility, increase of female fitness and partial selfing promote chloroplast capture. According to this model, chloroplast capture might have explanatory power for the phylogenetic incongruences observed in *Catasetum, Cycnoches* and *Mormodes*. Even though chloroplast pattern heritability in the Catasetinae is unknown, there is reliable evidence of its maternal inheritance in few orchid lineages such as *Anacamptis* Rich., *Doritis* Lindl., and *Phalaenopsis* Blume (Chang et al., 2000; Cafasso

et al., 2005). Moreover, all species of *Catasetum*, *Cycnoches*, and some of *Mormodes* are able to produce (though rare) intermixed inflorescences with pistillate and staminate flowers on the same individual (Gerlach, 2007; Gerlach & Pérez-Escobar, 2014). Therefore, they are facultative geitonogamous, as bees visiting male flowers might subsequently pollinate female flowers produced in the same inflorescence.

Field observations on pollination and floral fragrance profile studies indicate that hybridization is plausible in Euglossine bee pollinated orchid lineages such as *Catasetum*, *Cycnoches* and *Mormodes* (e.g., *Gongora* Ruiz & Pav., *Stanhopea* J.Frost ex Hook.: Williams & Whitten, 1983; Ramirez et al., 2011). These orchids produce a blend of volatile compounds, which attract male Euglossine bees. Pollination occurs while bees collect chemical compounds produced by specialised flower tissues (Gerlach & Schill, 1991). Species-specific production of floral blends and therefore attraction of a unique set of pollinator(s) has been accounted as an isolative reproductive barrier (Dressler, 1968; Ramirez et al., 2011) in Euglossine bee pollinated orchids. Nevertheless, sporadically intra-specific variation of the floral compound blend in several orchids such as *Stanhopea* (Williams & Whitten, 1983) and even in *Cycnoches* (Gregg, 1983) has been reported. Floral blend variation may result in the attraction of a set of pollinators that are shared by sympatrically occurring species with similar composition of the blend profile, hence favouring hybridisation to take place (Williams & Whitten, 1983). Little is known about specific pollinators of *Cycnoches*, but own observations of pollinator sharing between species (**Fig. 8**) and occurrence of species complexes (see "species delimitation" section of this *Introduction*) and polymorphic species might be the outcome of past and ongoing hybridisation processes.

Evolution of sexual systems in Catasetinae

The ample diversity of sexual systems is a remarkable trait of angiosperm lineages, and their lability and evolutionary transitions are key factors of lineage diversification (Barrett, 2013). Two sexual systems are predominant in Catasetinae, namely protandry and ESD (see "Reproductive systems in Catasetinae" section in *Introduction*), yet their mode and tempo of evolution in orchids have remained unknown. This is particularly true for ESD, which is an extremely rare sexual system in angiosperms (Renner, 2014) and for which there are very few studies available (e.g.

Renner et al., 2007). My detailed search of literature and *in-situ* and *ex-situ* observations on sexual systems in several taxa unveils an uneven distribution of protandry and ESD across Catasetinae. Interestingly, species having evolved ESD clustered in species rich lineages (e.g. *Catasetum*, *Cycnoches*), whereas protandrous species belonged to poorer species clades (e.g. *Clowesia*, *Dressleria*). Such uneven distribution is reflected in ML and Bayesian Ancestral State Reconstruction approaches, which strikingly reveals three independent origins of ESD (see Fig. 3 of **Chapter 6**), always derived from a protandrous ancestor. In addition, it endorses one of the equally parsimonious assumptions of Romero (1990), in which bisexual flowers (and hence plants with protandry) were proposed as the ancestral state of the last common ancestor of the "core Catasetinae" (see Taxonomic history of Catasetinae in *Introduction*).

Figure 8. Pollinator sharing in *Cycnoches guttulatum* (A) and *C. dianae* Rchb.f (B). Note the multiple *in-situ* visits (Panama) including pollinaria removal by the same bee species *Euglossa cyanura* Cockerell.

No positive correlation of the independent gains of ESD with ecological traits could be detected, contrary to what is observed in other angiosperm lineages with different sexual systems such as dioecy (e.g. Siparunaceae: Renner & Won, 2001). Nonetheless, the fact that in Catasetinae, species poor clades are associated with protandry and species rich lineages are related with ESD suggest that the latter might promote speciation in orchid lineages. ESD is favoured by natural selection when either male or female individual's fitness is affected by environmental conditions (Charnov & Bull, 1977; Korpelainen, 1998). Sex ratios in plants of Catasetinae with ESD are strongly biased depending on environmental conditions (Romero & Nelson, 1986; Zimmerman, 2011), with light intensity as a critical variable for sex determination (Gregg, 1982). Often Catasetinae orchids with ESD having more access to longer photoperiods produce female flowers and bigger pseudobulbs (hence they have bigger energetic resources) (Gerlach, 2007; Zimmerman, 2011). Surprisingly, these plants also bear capsules of considerable size, compared with those from closely related lineages (e.g. *Clowesia*, *Dressleria*) with protandry (Pérez-Escobar, *pers. obs.*; Salazar G., *pers. com.*). For instance, capsules of *Cycnoches chlorochilon* Klotzsch (also a member of Catasetinae with ESD) have on average three times more seeds (3,770,000) than the capsules of *Cymbidium tracyanum* L.Castle (850,000), an adichogamous species closely related to Catasetinae (Arditti & Ghani, 2000). Unfortunately, the lack of morphological data for all Catasetinae (no representative herbarium specimens with capsules found) precluded the statistical testing of this assumption during my research. Future studies should involve extensive field work to understand more about the reproductive biology of Catasetinae orchids, which ultimately will further enlighten reproductive systems' lability and their evolutionary transitions.

Biogeography of *Cycnoches*

Three nuclear and three chloroplast loci recovers strongly supported internal phylogenetic relationships of *Cycnoches* and provides a solid phylogenetic framework for Ancestral Area Estimation and biogeographical hypothesis testing. Absolute time estimates reveal that *Cycnoches* diversified during the late Miocene, around 6 MYA in the lowland wet forests of the Amazonian region (Fig. 3, **Chapter 7**), at a time when the Central and Northern Andes ranges already peaked elevations of 4500 m (Hoorn et al.,

2010). Andean uplift is one of the most important orographic events in the geographic history of South America (Hoorn et al., 2010; Luebert et al., 2011), because it had a profound impact on the regional landscape (Hoorn, 1994; Hoorn et al., 1995). Andean orogeny was a constant geological process with discrete periods of time of accelerated building (Hoorn et al., 1995; Ghosh et al., 2006; Antonelli et al., 2009) that greatly altered the climatic patterns of the subcontinent (Hoorn et al., 2010), brought forth several novel habitats at mid and high elevations (Hughes & Eastwood, 2006; Moore & Donoghue, 2007) and lastly, it settled a geographic barrier that isolated populations in either side of the range (Pirie et al., 2006; Antonelli et al., 2009). The latter is particularly evident in some clades of terrestrial plant lineages such as the Neotropical Rubiaceae and Annonaceae, which show clear east-west Andes or lowland vs. highland restrictive disjunctive distributions (Pirie et al., 2006; Antonelli et al., 2009). Nevertheless, the role of the Andes as an isolative barrier for lowland, epiphytic anemochorous angiosperm lineages (a prominent component of the Neotropical flora; Kreft et al., 2004; Funk et al., 2007), is still poorly understood.

My absolute ages estimates and AAE reveals a younger origin of *Cycnoches* compared with Andean paleo-altitude, suggesting trans-Andean dispersals from the Amazonas region towards Central America and Choco. Hence, this mountain range do not represent an important physical, isolative barrier neither for *Cycnoches* and probably nor for other epiphytic, anemochorous lineages. Orchid seeds, often called dust-like seeds (Dressler, 1993; Arditti & Ghani, 2000), are very small sized (from 0.05 to 6 mm) and their testae often presents a highly elaborated morphology, thus allowing them to float in the air for prolonged periods and distances. These characteristics facilitate long distance dispersal and therefore rare trans-Andean migrations were likely to occur in Neotropical orchids. My results are in line with biogeographic patterns observed in other epiphytic, wind-dispersed lineages such as bromeliads. A unique study on historical biogeography of bromeliads (Givnish et al., 2011) revealed long distance trans-Andean dispersals in the Bromelioideae from the Brazilian Shield towards Central America around 6 MYA.

Changes in diversification rates are often associated with the evolution of novel morphological traits that promote speciation (e.g. nectar spurs and heterospory in seed plants; Bateman & DiMicehele, 1994; Hodges, 1997). In *Cycnoche*s however, radiations in areas such as Central America (~ 1.5 MYA) might be associated rather with the

colonization of regions followed by habitat specialization. Similar rapid diversifications after colonization have been observed in other terrestrial angiosperm clades such as the Adoxaceae and Valerianaceae (Moore & Donoghue, 2007) and more recently in the taxon *Bartsia* L. (Orobranchaceae; Uribe-Convers & Tank, 2015). In the latter lineage, the establishment and diversification in South American Andean highlands of representatives derived from a Eurasian LCA, correlates with Andean uplift ages that created new habitats similar to those observed in Alpine landscapes. Diversification because of microhabitat specialization is known from mid-high altitude terrestrial lineages, but my research reports for first time this diversification mode for epiphytic lowland clades. The phytosociological composition of Central American and Amazonian lowland wet forests are divergent (Cuatrecasas, 1958; Rangel-Ch et al., 1997; Lentz, 2000), but the environmental conditions such as rainfall and relative humidity that are crucial for epiphytism (Kreft et al., 2004) might have been similar during the diversification of *Cycnoches*, hence facilitating the radiation of this lineage in both Central America and Amazonia (Fig. 3 of **Chapter 7**).

Species delimitation in *Cycnoches* using Next Generation Sequencing technologies

Because morphology does not provide useful information to delimitate species in the *C. egertonianum* complex, I investigated floral fragrance composition and restriction-site-associated genomic markers (obtained via high throughput sequencing) to better understand species boundaries in this complex. For the fragrance profile analysis, I have collected so far 200 samples from 40 individuals (**Appendix S1**), which have been analyzed at the laboratory of Prof. Stefan Dötterl (Universität Salzburg), with the assistance of Dr. Irmgard Schäfer. In addition, samples of 35 individuals have been sequenced using Genotyping by Sequencing approach (GBS) at the laboratory of Dr. Frank Blattner (IPK – Gatersleben), where I learned to analyse the results using bioinformatics tools (i.e. PyRAD: Eaton, 2014) (**Appendix S2**). I am still working on the analysis of the output data of both the fragrances analyses and the GBS, which are thus only presented as appendixes of this dissertation, and will be employed in population structure and phylogenetic analyses for further publications.

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APPENDIX

Table S1. List of species of which fragrance profiles have been sampled (marked with "+"). Members of the *Cycnoches egertonianum* complex are indicated in boldface.

Species	Fragrance analysis
***Cycnoches amparoanum* Schltr.**	-
Cycnoches aureum Lindl. & Paxton	+
Cycnoches barthiorum G.F.Carr & Christenson	+
Cycnoches chlorochilon Klotzch	+
***Cycnoches densiflorum* Rolfe**	-
***Cycnoches dianae* Rchb. f.**	+
***Cycnoches egertonianum* var. *egertonianum* Bateman**	+
***Cycnoches egertonianum* var. *viride* Lindl.**	+
***Cycnoches guttulatum* Schltr.**	+
Cycnoches haagii Barb.Rodr.	+
Cycnoches herrenhusanum Jenny & G.A. Romero	+
Cycnoches lehmannii Rchb.f.	+
Cycnoches manoelae P.Castro & Campacci	+
***Cycnoches pachydactylon* Schltr.**	-
Cycnoches peruvianum Rolfe	+
***Cycnoches powellii* Schltr.**	-
***Cycnoches rossianum* Rolfe**	+
***Cycnoches stenodactylon* Schltr.**	-
Cycnoches ventricosum Bateman	+
Cycnoches warszewiczii Rchb.f.	+

Table S2. List of sequenced samples of *Cycnoches* individuals using GBS approach.

Putative species identity	Accession/voucher
Cycnoches aureum Lindl. & Paxton	BGM 2013/2503w
Cycnoches barthiorum G.F.Carr & Christenson	BGM 2012/1476
Cycnoches chlorochilon Klotzch	BGM 2013/2436w
Cycnoches dianae Rchb. f.	*Pérez & Gerlach 1468*
Cycnoches dianae Rchb. f.	*Pérez & Gerlach 1470*
Cycnoches egertonianum Bateman	Perez 1509
	Perez, Machorro & Rodriguez 1522
	Perez, Martinez, Castillo 1535
	Pérez 1463
Cycnoches cf. *egertonianum* Bateman	BGM 2013/2483w
Cycnoches egertonianum var. *viride* Lindl.	BGM 2012/1471
	Perez, Machorro & Rodriguez 1534
	Cash & Perez 1505
Cycnoches guttulatum Schltr.	BGM 2013/2507w
	BGM 2013/2500w
	Pérez & Gerlach 1478
	Pérez & Gerlach 1476
	BGM 2013/2505w
Cycnoches cf. *guttulatum* Schtrl.	BGM 2012/2124
Cycnoches herrenhusanum Jenny & G.A. Romero	BGM 2012/1473
Cycnoches aff. *pachydactylon* Schltr.	*Pérez & Gerlach 1471*
Cycnoches pachydactylon Schltr.	*Pérez & Gerlach 1469*
Cycnoches peruvianum Rolfe	BGM X/1351
Cycnoches rossianum Rolfe	BGM 2014/1832w
	Gomez & Perez 1496
	Treminio & Perez 1497
	Pérez & M.A. Blanco 1467
	Pérez & M.A. Blanco 1466
Cycnoches sp.	Gomez & Perez 1498B
Cycnoches sp.	Gomez & Perez 1498A
Cycnoches sp.	BGM 2013/2502w
Cycnoches sp.	BGM 2013/2504w
Cycnoches sp.	*Perez et al. 1491*
Cycnoches sp.	*Perez et al. 1492*
Cycnoches sp.	*Perez 1510*

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September 2010: Lead researcher of the project "Orchidologic inventory of cloud forests from Dapa, Yumbo municipality", funded by Dapaviva Environmental Foundation.

Peer-Reviewed Publications

Pérez, O.*; Chomicki, G.*; Condamine F.L.; Matzke, N.J.; Silvestro, D.; Antonelli, A. (*submitte*d) Mountain uplift triggered the diversification of Neotropical orchids.

Feldberg, K.; Vána, J.; Krusche J.; Kretschmann, J.; Patzak, S. **Pérez, O.**; Rudolph, N.; Seefelder N.; Schäfer-Verwimp, A.; Long, D.; Schneider, H.; Heinrichs, J. (*in rev.*) A phylogeny of Cephaloziaceae (Jungermanniopsida) based on nuclear and chloroplast DNA markers.

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Books

Kolanowska, M.; **Pérez, O.**; Parra, E.; Szlachetko, D. (2011) An illustrated field guide to the orchids of the Yotoco Forest Reserve (Colombia). Fundacja Rozwoju Uniwerytetu Gdanskiego. Gdansk, Poland. 293 p. (available at https://www.koeltz.com/product.aspx?pid=202944)

Monographs

Hágsater, E.; Santiago, E.; Sánchez, L.; **Pérez, O.** *et al* (+9 authors) (2010) Icones Orchidacearum Fascicle 13: The genus *Epidendrum*, part 9. Herbario AMO, México. (available at: http://www.herbarioamo.org/index_archivos/Fascicle13.pdf)

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Non-Peer Reviewed Publications

Gerlach, G.; Pérez, O. (2014) Looking for missing swans: Phylogenetics of *Cycnoches* (Catasetinae: Orchidaceae). *Orchids* 81: 434-437.

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Ortiz, P.; **Pérez, O.**; Parra, E. (2009) A new and interesting species of *Lepanthes* (Orchidaceae) from Colombia. *Orquideología* 26(2): 55-62

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$4,500. Organization for Tropical Studies (OTS) (2012). Scolarship for participation on the course of "Systematics of Tropical Plants", organized by the OST

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Contributed Research Presentations

Pérez, O. Phylogenetics and Biogeography of Darwin's favorite orchids. EES Conference - 2015. (LMU-Munich).

Pérez, O. Quantifying divergent evolution between nuclear host and chloroplast endosymbionts. Genomics meets Phylogenetics workshop - 2015. (CAS-Munich).

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University of Leipzig, Germany (2015 - three weeks): *Programming for Evolutionary Biology Workshop* (organized by Katja Nowick and Rui Faria)

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Leipzig, Germany (2016 - five days): *A beginner's guide to RNA-seq data analysis course* (organized by ecSqQ Bioinformatics GmbH)

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