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Reticulate evolutionary history underpins a revised generic circumscription of *Paphiopedilum* (Orchidaceae): insights from integrative phylogenomics and historical biogeography

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Abstract

Background The taxonomy of *Paphiopedilum*—a diverse group of slipper orchids—has long posed challenges due to high morphological variability and conflicting phylogenetic signals. Despite intensive research, a clear and stable infrageneric classification remains unresolved.

Results We present an integrative phylogenetic framework based on multiple nuclear low-copy genes (*XDH*, *PHYC*, *LFY*, *RAD51*, *ACO*, *DEF4*), ITS and plastid (*matK)* sequences, supported by morphological and biogeographical data. Our analyses consistently recovered monophyletic clades corresponding to both historically recognized and newly proposed lineages. Phylogenetic incongruence observed both between different nuclear loci and between nuclear and plastid phylogenies suggests a reticulate evolutionary history shaped by ancient hybridization events and incomplete lineage sorting. Biogeographic reconstructions place the genus' origin in mainland of Southeast Asia during the Miocene, with subsequent radiation influenced by climatic and geological events. As a result, we propose a revised classification recognizing three genera—*Parvisepalum*, *Brachypetalum*, and *Paphiopedilum* s.str.—with the latter subdivided into six subgenera.

Conclusions This study provides the most comprehensive phylogenetic and taxonomic treatment of *Paphiopedilum* to date. Our framework reflects evolutionary relationships more accurately than previous systems and offers a stable basis for future research in orchid systematics, conservation, and evolution.

Keywords Orchidaceae, *Paphiopedilum*, Phylogenetics, Slipper orchids, Hybridization, Taxonomy, Biogeography, Molecular systematics

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Introduction

For the past 200 years, many taxonomists have focused their attention on *Cypripedium* and related genera. Linnaeus (1753), who distinguished the genus, recognized only two species in it and numerous varieties. One of these species, *Cypripedium bulbosum*, was transferred in 1842 by Oakes to the genus *Calypso*, described by Salisbury in 1808 in the monandrous orchids. Lindley (1840) proposed 22 species, all placed in *Cypripedium* [1]. Reichenbach f. (1854) suggested the consolidation of all tropical American lady's slipper orchids into a single genus, *Selenipedium* [2]. In 1886 Pfitzer established the genus *Paphiopedilum* to accommodate species with conduplicate leaves [3]. Three years later Rolfe (1896) divided Pfitzer's group into two separate genera [4]. He placed all species with a three-chambered ovary in *Phragmipedium*, leaving those with a one-chambered ovary in *Paphiopedilum*. Pfitzer (1898) merged the *Phragmipedium* and the *Paphiopedilum* groups into a single taxon under the priority name *Paphiopedilum*, with American species classified as a group [unranked] *Phragmopedilum* [5]. For over 100 years, the division of lady's slipper orchids into four genera was widely accepted. To highlight the differences in morphological structure, diandrous orchids were combined into a taxon of different ranks (Diandra, Pleonandra, Cypripedioideae), in opposition to monandrous orchids.

Cypripedioideae constitutes a homogenous taxon, clearly distinct from other representatives of Orchidaceae. They encompass five genera, characterized by the presence of two fertile anthers and trap-like flowers, an adaptation for insect pollination. Due to the presence of two fertile stamens in Cypripedioideae, they are considered a more primitive taxon than monandrous orchids [6–8]. However, molecular data indicate that the more basal taxon is the subfamily Vanilloideae, which has a single fertile stamen [9, 10]. The column of cypripedioid orchids is distinct, with a large stigmatic shield having three differently sized and shaped lobes. Szlachetko (1995), based on leaf type and stem structure, divided Cypripedioideae into two subtribes: Cypripediinae (*Cypripedium* and *Selenipedium*) and Phragmipediinae (*Mexipedium*, *Paphiopedilum*, and *Phragmipedium*) [8]. Molecular studies indicate that *Mexipedium* is a sister taxon to *Phragmipedium* [11] and together with the genus *Paphiopedilum* form a monophyletic group. The evolution of the one-chambered ovary in *Mexipedium* is a case of convergent evolution and likely to have occurred independently from *Paphiopedilum*. The genera *Paphiopedilum* and *Phragmipedium* have evolved in parallel. Both taxa exhibit certain analogies. The most primitive subgenera are *Parvisepalum* in *Paphiopedilum* and *Micropetalum* in *Phragmipedium*. Both subgenera have flowers with short sepals and petals, and the margins of the lip are folded inward, similar to *Selenipedium* and many species of *Cypripedium*.

Among Cypripedioideae, both tropical epiphytes and terrestrial perennials in the subantarctic climate zone are found. *Selenipedium* and *Phragmipedium* occur in South and Central America. On the other hand, the genus *Mexipedium* is an endemic taxon, occurring only in the state of Oaxaca in Mexico. *Cypripedium* is found in the temperate and subtropical zones of the Northern Hemisphere, while *Paphiopedilum* is restricted to the Himalayas and the zone stretching from southern China through Malaysia to Guadalcanal. One species, *P. druryi*, is only found in southern India (Kerala State), approximately 2000 km away from the locations of other species.

Paphiopedilum, a diverse genus of slipper orchids, has long posed a taxonomic challenge due to its high morphological variation and inconsistent molecular phylogenetic signals. Despite extensive research, its infrageneric classification remains unsettled. These topological incongruences highlight the complexity of *Paphiopedilum* evolution and suggest possible instances of reticulate evolution or incomplete lineage sorting. As such, this genus remains a model system for investigating the limits of phylogenetic resolution and the impact of different genomic data types on orchid taxonomy. The division of the genus *Paphiopedilum* into subgenera and sections are based mainly on floral morphology and types of leaves [12–17]. Brieger (1971) divided the genus into four subgenera: *Polyantha* Pfitz., *Brachypetalum* Hall., *Paphiopedilum* and *Barbata* Kraenzl.. Subgenus *Polyantha* consists of species with multi-flowered inflorescences [12]. Subgenus *Brachypetalum* included only five species with large broad elliptic petals. There were *P. concolor* (Bateman) Pfitz., *P. bellatulum* (Rchb.f) Pfitz., *P. delenatii* Guill., *P. godefroyae* (Hall.) Pfitz., and *P. niveum* (Rchb.f.) Pfitz. In turn, the subgenera *Paphiopedilum* and *Sigmatopetalum* were separated based on the morphology of the staminode, petals and leaves.

Karasawa and Saito (1982) in their revision of the genus additionally used the chromosome numbers and density of stomata, and with high precision made differentiation between some subgenera [13]. *P. delenatii* from subgenus *Brachypetalum* sensu Brieger (1971) and two newly—at that time—described species, *P. armeniacum* and *P. micranthum*, were transferred to the newly proposed subgenus *Parvisepalum* Karas. & Saito. It differs from *Brachypetalum* in several features, as granulous pollinia, elongate stigmata, occurrence of underground stolons and the morphology of the perianth to mention the most important [13]. One of the sections (*Cochlopetalum*) of multi-flowered subgenus *Polyantha*, were raised to the rank of the subgenus *Cochlopetalum* (Hallier f.) Karas. & Saito, which differs from *Polyantha* in the number of chromosomes, morphology of the perianth segments

and flowers that open successively opposite to simultaneously in *Polyantha* sensu stricto. Karasawa and Saito (1982) narrowed the concept of subgenus *Barbata* sensu Brieger (1971) only to the species with tessellated leaves, well developed laterals ears of the lip and chromosome number (2n) ranging from 28 to 42 [13]. They adopted name *Sigmatopetalum* Hallier f. for this subgenus, in accordance with ICBN (International Code of Botanical Nomenclature).

Cribb (1987) recognized only two subgenera: *Brachypetalum* (Hallier f.) Pfitzer (including sections *Concoloria* and *Parvisepalum*) and *Paphiopedilum* [14]. The latter was divided into five sections: *Paphiopedilum, Barbata* (Kraenzl.) V.A. Albert & B. Pettersen, *Cochlopetalum* Hallier f. *ex*. Pfitzer, *Pardalopetalum* Hallier f. & Pfitzer, and *Coryopedilum* Pfitzer. The subgenus *Paphiopedilum* sensu Cribb is characterized by flowers in which the petals are tapering, oblong or spathulate and more than twice as long as broad, and a lip in which only the side-lobes are incurved [15]. The different view concerning infrageneric classification of the genus has been proposed by Braem (1988) and Braem & Chiron (2003). In their approach, the authors followed Karasawa and Saito (1982) and divided subgenus *Paphiopedilum* sensu Cribb into the following subgenera: *Paphiopedilum*, *Sigmatopetalum* Hallier f. *ex* Karas. & K. Saito, *Polyantha* (Pfitzer) Brieg. and *Cochlopetalum* (Hallier f. *ex* Pfitzer) Karas. & K. Saito [16, 17].

The verification of the phylogeny of the genus *Paphiopedilum* occurred with the emergence of phylogenetic analyses based on DNA sequences. The first of these analyses were based on the nuclear ITS1-5.8S-ITS2 region (ITS—Internal Transcribed Spacer). Cox et al. (1997) based on molecular analysis of ITS suggested division of *Paphiopedilum* into three subgenera: *Parvisepalum* Karas. & K. Saito (= sect. *Parvisepalum* sensu Cribb 1987), *Brachypetalum* Karas. & K. Saito (= sect. *Concolaria* sensu Cribb 1987) and *Paphiopedilum.* The latter was splitted into four sections. Cox et al. (1997) have included sect. *Coryopedilum* within *Pardalopetalum* [11]. Cribb (1998) in the second edition of his monograph of *Paphiopedilum* follow Cox et al. (1997) division except the combination of these two sections [15]. Recently published molecular phylogenetics of *Paphiopedilum* clarify relationships between sections of the nominal subgenus of *Paphiopedilum* (sensu Cribb) and re-create section *Coryopedilum* [18]. The relationships between sections within the subgenus *Paphiopedilum* were further validated by plastid DNA analysis, revealing the existence of two evolutionary lineages — one encompassing single-flowered species (sections *Barbata* and *Paphiopedilum*) and the other one comprising multi-flowered species (sections *Coryopedilum*, *Pardalopetalum*, and *Cochlopetalum*) [18]. Górniak et al. (2014) [19] demonstrated, based on nuclear data (ITS, *XDH*—gene encoding xanthine dehydrogenase), the existence of a new lineage, a monotypic one, containing *P. canhii.* Other nuclear marker analyses support this phylogenetic position (*ACO*—gene encoding 1-aminocyclopropane-1-carboxylate oxidase) or suggest its belonging to the nominal section (*DEF4*—DEFICIENS-like gene 4 and *RAD51*—gene encoding DNA repair protein) [20]. On the other hand, plastome analysis shows that *P. canhii* is sister to the multi-flowered sections [21]. Previously, Braem and Gruss (2011) proposed a new subgenus *Megastaminodium* Braem & O. Gruss to accommodate this species [22].

It is important to note that the currently accepted phylogeny, reconstructed based on plastid data, finds support (except *P. canhii*) in morphological data [18], although it is not corroborated by most nuclear genes studied to date. The *XDH* marker [19] indicates that section *Paphiopedilum* and all three multi-flowered sections are sister groups, a result consistent with the *LFY* marker (gene encoding LEAFY protein) [23] except for section *Cochlopetalum*, where *LFY* supports *Cochlopetalum* as a sister group to section *Barbata*. Conversely, *RAD51* [23] suggests that section *Cochlopetalum* is sister to all other sections of the subgenus *Paphiopedilum*. None of the mentioned nuclear markers (*LFY*, *XDH*, and *RAD51*) support the monophyly of subgenus *Paphiopedilum*, and although the *ACO* and *DEF4* nuclear markers strongly support it [23], they do not resolve relationships within this taxon (polytomy). It is noteworthy that the incongruence in tree topology among different nuclear markers [19, 23] within subgenus *Paphiopedilum* pertains to sections *Barbata* and *Cochlopetalum.*

Recently Górniak et al. (2021) showed that other the low-copy nuclear protein-coding gene (*PHYC)* support morphological characteristics and plastid data analysis. The results obtained based on incongruent topologies of phylogenetic trees could indicate ancestral hybridization as the cause of nuclear marker incongruence [24]. Moreover, there exists a systematic uncertainty regarding four taxa (*P. canhii, P. fairrieanum, P. hirsutissimum,* and *P. rungsuriyanum*) due to their atypical morphologies, which do not conform to the established subgeneric classifications. Previously molecular analyses fail to explain this issue [20, 23, 25].

The aim of this study is to establish a natural classification of the genus *Paphiopedilum* and to determine the systematic position of the aforementioned species based on molecular analyses and morphological characteristics.

Materials and methods

DNA isolation, amplification, and sequencing

Plant specimens used in this study originated from the private collection of Professor Dariusz L. Szlachetko. No wild-collected specimens requiring special permits

were used. The specimens were originally obtained from both private botanical collections and scientific institutions (botanical gardens). Original sources included Royal Botanic Gardens, Kew (UK); Herrenhäuser Gärten, Berggarten (Germany); Orchideen Popow (Germany); Chen Li Plants (China); In Vitro, T. Kusibab (Poland); Orchidées du Sénat, P. Bertaux and P. Sauvetre (France); Riboni Collection (Italy); Z. Konsik (Poland); and the private collection of Canh C.X. (Vietnam). In total, 232 specimens representing 95 species were sequenced. The original source of each specimen is indicated in the supplementary materials accompanying the respective entry. Each specimen was properly identified by Prof. Dariusz L. Szlachetko, photographed, and documented following standard botanical protocols. In addition, molecular identification was carried out using the Basic Local Alignment Search Tool (BLAST) algorithm. Genomic DNA was obtained from 20 mg dry desiccated in silicagel [26] parts of leaves using a DNA Mini Plant Kit (A&A Biotechnology, Poland). Detailed laboratory procedures, including the use of primers, specific conditions for Polymerase Chain Reaction (PCR), purification, and sequencing for ITS, low-copy nuclear *XDH*, and plastid *matK* (maturase K) genes, were outlined in a separate publication [19]; protocols for *LFY*, *RAD51*, *ACO*, and *DEF4* followed those described by Guo et al. [23], while methods for the low-copy nuclear gene *PHYC* are described in Górniak et al. [24]. The PCR reaction employed Dream Taq PCR Master Mix (2x) (Thermo Scientific). In instances where nucleotide heterogeneity was observed, an additional step of DNA cloning was performed using the pJET1.2 vector (Thermo Scientific CloneJET PCR Cloning Kit). Competent cells were prepared using *Escherichia coli* cells and calcium chloride ($CaCl_2$). PCR amplification was carried out using pJet1.2_F and pJet1.2_R primers provided in the kits. The High Pure PCR Product Purification Kit (Roche Diagnostic GmbH, Germany) was used for purifying PCR products, following the manufacturer's protocol. Sequencing was conducted using the Big Dye Terminator v 3.1 Cycle Sequencing Kit (Applied Biosystems, Inc., ABI, Warrington, Cheshire, UK) with the same primers used in the amplification step. Accession numbers for the DNA sequences analyzed in this study were deposited in the GenBank database through sequence submissions. For each marker, accession numbers were retrieved as continuous ranges based on the order of submission: *ACO*: PV684576–PV684582, *DEF4*: PV684583–PV684586, *RAD51*: PV684587–PV684590, *LFY*: PV684591–PV684593, *PHYC*: PV693818–PV693830, *XDH*: PV704403–PV704478, PV941805–PV941806, *matK*: PV704479–PV704579, PV941798–PV941804, ITS: PV704793–PV704897, PV938549–PV938557. These accession numbers correspond to sequences generated for this study and are publicly available in the NCBI GenBank database. GenBank accession numbers for each specimen can be found in Additional File 1, Table S1.

Molecular phylogenetics

Finch TV (Geospiza) was used to edit the sequences, and the two complementary strands were assembled by using AutoAssembler (ABI). All new data matrices (ITS, *XDH*, *PHYC*, *matK*) were aligned using MAFFT [27], v. 7 and then analyzed by SeaView v. 4 [28]. Four other nuclear low copy genes (*LFY, RAD51, ACO*, *DEF4*) matrices were downloaded from repository [23]. Using the Seaview v. 4 program, the sequences of newly sequenced species, namely *P. canhii*, *P. fairrieanum*, and *P. rungsuriyanum*, were manually aligned to those matrices. For all sequences obtained from public repositories, including GenBank, corresponding GenBank accession numbers are provided in Additional File 1, Table S1. Representatives of all subgenera from the genus *Paphiopedilum* were used in the analyses. *Mexipediumm xerophyticum* and several *Phragmipedium* species were used as an outgroup. Eight individual data matrices were analysed – ITS, *XDH, PHYC,* plastid (*mat*K), *LFY, RAD51, ACO* and *DEF4*. To improve phylogenetic resolution, the seven nuclear markers were grouped into three datasets based on specimen origin and topological compatibility. There were: 1. ITS/*XDH*, 2. *LFY/RAD51/ACO*/*DEF4*, 3. *ACO*/*DEF4*/*PHYC*. The first and second groups comprised markers sequenced from the same specimens. In particular, the first and second groups were assembled to enhance phylogenetic signal and clarify the position of *Paphiopedilum fairrieanum*, *P. rungsuriyanum*, and *P. hirsutissimum*, which showed unstable placements in single-marker analyses—typically forming polytomies or weakly supported clades within subgenus *Paphiopedilum*. The third group included the only markers without topological incongruence and was used to resolve relationships among subgenera. The datasets supporting the conclusions of this article are available in the Figshare repository, [https://doi.org/10.6084/m9.figshare.28981424]. Phylogenetic reconstruction for all data set was undertaken using Bayesian inference using MrBayes 3.2 [29]. Substitution models were selected as the best fitting model by Akaike information criterion as implemented in MrModeltest v. 2.2 [30] (Additional File 1, Table S2). The posterior probabilities (PP) of clades were estimated by sampling trees from the PP distribution using MCMC (Markov chain Monte Carlo) simulations. Six chains were run for 10,000,000 generations. Trees were sampled every 1000 generations. Trees were checked for stability, which happened around the 100,000th chain (average standard deviation of split frequencies was < 0.01). The first 7500 trees were discarded as the burn-in. Nodes with posterior probability (PP) values equal to or greater than 0.95 were

considered significant, while those with PP values below 0.95 were considered non-significant.

To test the hybridization hypothesis, the PhyloNet software v. 3.8.0 [31, 32] with the *MCMC_GT* (Markov Chain Monte Carlo using Gene Trees) analysis option was selected [33]. *MCMC_GT* infers phylogenetic networks using Bayesian MCMC from gene tree topologies using a multispecies network coalescent framework (MSNC). MSNC incorporates hybridization in addition to incomplete lineage sorting (ILS). The length of the MCMC chain was 4,000,000 with 400,000 of iterations in burn-in period. The sample frequency was 1000. The maximum number of reticulation nodes in the sampled phylogenetic networks was the default value—infinity. Network/Trees generated by PhyloNet were visualized by Dendroscope v. 3.6.3 [34]. The trees obtained from the Bayesian analysis of individual markers were used in the PhyloNet. Clades with PP (posterior probability) values of 0.97–1.0 were maintained on the trees. The topology of the trees in newick format was as follows: [*XDH*] (((((Cor,Par,Coch),Pap),M),S,B),P); [*RAD51*] (((((Cor,Par),Pap,S),M),C,B),P); [LFY] ((((((Cor,Par),Pap),M),(Coch,S)),B),P); [*ACO*] ((((Cor,Par),Coch,Pap,S,M),B),P); [*DEF4*] ((((Cor,Par),Coch,Pap,S,M),B),P); [*PHYC*] (((Cor,Par,Coch),(Pap,S),M),B,P); [ITS] (((Cor,Par),Coch,Pap,S,M,B),P); [Plastid] (((((Cor,Par,Coch),M),(Pap,S)),B),P);. Nodes presented in newick format were as follows: Cor, Par— subg. Polyantha; Coch—subg. *Cochlopetalum*.; S—subgen. *Sigmatopetalum*; Pap—subgen. *Paphiopedilum*; M—subgen. *Megastaminodium* B—subgen. *Brachypetalum*; and P—subgen. *Parvisepalum*.

Ancestral range estimation

The biogeographic analysis was based on the combined *ACO*/*DEF4*/*PHYC* matrix. This was the only matrix for which the analysis was consistent with the species tree obtained based on conflicting phylogenetic tree topologies in the PhyloNet software. The analysis was conducted in two stages. In the first stage, phylogenetic trees were obtained using the BEAST program (Bayesian Evolutionary Analysis Sampling Trees). Subsequently, the actual biogeographic analysis was performed using the RASP 4.4 (Reconstruct Ancestral State in Phylogenies) [35]. The Bayesian strict molecular clock approaches implemented in BEAST v. 1.8.1 [36] were used. For each of the three genes, the previously obtained model of evolution was applied. The birth–death process was chosen as the speciation process. Two runs were performed in BEAST, with 100 million generations each and a sampling frequency of 1000. Tracer v. 1.6 [37] was used to analyse Log files to assess the convergence. The combined effective sample sizes for all the chosen parameters were larger than 200. All the obtained trees were combined with LogCombiner v. 1.8.1 [36], with a burn-in of 25%. Finally, the maximum clade credibility tree (MCCT) was produced using TreeAnnotator v. 1.8.1 [36]. The obtained phylogenetic trees (150,002) and the MCC tree served as the starting point for the analysis in the RASP 4.4 [35]. For the purposes of the analysis, six geographic regions were coded, two of which referred to outgroup taxa (*Mexipedium* and *Phragmipedium*). Coding system represent the current distribution of the genus *Paphiopedilum* as follows: A, Qinghai-Tibetan Plateau (QTP) and Hengduan Mountains [38, 39]; B, Southern of China; C, Mainland of Southeast Asia; D, Malay Archipelago; E, Mesoamerica (*Mexipedium* only); and F, South America (*Phragmipedium* only). The ancestral geographical ranges of *Paphiopedilum* were reconstructed using S-DEC (Statistical Dispersal-Extinction-Cladogenesis) [40, 41] and BAYAREALIKE + J (J—Jump dispersal/Founder-event speciation) model implemented in R package'BioGeoBEARS'1.1.1[42],executed in RASP 4.4 [35]. The latter was selected based on the corrected Akaike Information Criterion (AICc), which identifies the best-fitting model. Additionally, the p-value of the Likelihood Ratio Test (LRT) was used to assess whether the inclusion of parameter J significantly improved model likelihood, allowing for rejection of the null hypothesis that models with and without J fit the data equally well (Additional File 1, Table S3).

Morphological analyses

We conducted studies of morphological traits on live material from the University of Gdansk collection and on flowers preserved in Kew Mix and Copenhagen Mix deposited in the UG herbarium (UGDA). For the purpose of our work, we did not consider herbarium material. The initial phase of the study consisted of screening with a stereomicroscope. When more detailed studies were required, we used electron microscopy available at the Bioimaging Laboratory of the University of Gdansk. The photographic documentation and iconography resulting from the above research is deposited in the herbarium archive of the University of Gdansk (UGDA). The taxa distinguished were expected to form molecularly and morphologically coherent groups, exhibiting a clear degree of discontinuity from other related taxa. In our view, the lack of trait continuity provides a rationale for assigning a group taxonomic status at a higher rank, such as a genus—as demonstrated in the case of *Paphiopedilum*.

We began our research by examining general morphological traits, including the type and sequence of flower development within the inflorescence and leaf blade colouration, followed by more detailed floral characteristics. Leaf colouration (tessellated/marbled vs. plain green), previously considered diagnostic for lower-ranking taxonomic units (e.g., sections), turned out to

be ecologically driven—particularly under shaded forest floor conditions—and was found across unrelated groups. In this orchid group, which exhibits trap flowers, the architecture of the lip plays a central role in the pollination mechanism. Key traits include the general shape of the lip (calceolate vs. not calceolate), the inward curvature of the lip margins, and the presence or absence of lateral lobes and auricles—all of which contribute to insect entrapment. Equally important is the form and proportion of sepals to petals, which likely act as long-range visual attractants for pollinators. The next stage of the study involved the removal of perianth segments and the detailed examination of generative structures. Particularly significant were the following qualitative characters: stigma (smooth or mammillate), pollinia (viscid or granular), stalk anther (short or long).

Results

Phylogenetics analysis

Statistics for one of the most parsimonious trees are shown in Additional File 1, Table S4. All individual and combined phylogenetic analyses consistently confirmed the monophyly of the recognized subgenera: *Parvisepalum*, *Brachypetalum*, *Sigmatopetalum*, *Paphiopedilum*, *Cochlopetalum*, *Polyantha*, and the monotypic *Megastaminodium*. However, substantial incongruence was observed among the phylogenies inferred from different markers, particularly regarding the relationships between subgenera.

Parvisepalum and *Brachypetalum* were consistently recovered as successive sister clades to all other subgenera (= subgenus *Paphiopedilum* sensu Cribb) in analyses based on *matK* (plastid, Fig. S1 in Additional file 2), *LFY* (Fig. S2 in Additional file 2), *ACO* (Fig. S3 in Additional file 2), and *DEF4* (Fig. S4 in Additional file 2) markers. However, in ITS (Fig. S5 in Additional file 2), *XDH* (Fig. S6 in Additional file 2), *RAD51* (Fig. S7 in Additional file 2) and *PHYC* (Fig. S8 in Additional file 2) trees, *Brachypetalum* often appeared in polytomies.

In the combined ITS/*XDH* analysis (Fig. S9 in Additional file 2), monophyly of all subgenera was supported, with improved support values compared to single-marker trees. In this tree, *Polyantha*, *Cochlopetalum* (PP = 1.0), and *Paphiopedilum* (PP = 1.0) formed a well-supported clade, sister to *Megastaminodium* (PP = 0.96), while *Sigmatopetalum* and *Brachypetalum* appeared in a polytomy, each supported individually (PP = 1.0).

The *ACO* (Fig. S3), *DEF4* (Fig. S4), and ITS (Fig. S5) markers strongly supported the monophyly of each subgenus (PP = 1.0), but failed to resolve their mutual relationships, resulting in polytomies. Analyses based on *LFY* (Fig. S2), *RAD51* (Fig. S7), *XDH* (Fig. S6), and *PHYC* (Fig. S8) revealed conflicting placements of *Sigmatopetalum* and *Cochlopetalum*. In *LFY*, these two subgenera formed a well-supported sister relationship (PP = 1.0). In contrast, *RAD51* placed *Cochlopetalum* as sister to *Brachypetalum* (PP = 0.86), and *Sigmatopetalum* appeared in a polytomy with *Paphiopedilum* and *Polyantha* (PP = 1.0). The *XDH* tree showed *Sigmatopetalum* as strongly supported (PP = 1.0), forming a polytomy with *Brachypetalum*. The *PHYC* tree (Fig. S8) supported the monophyly of major subgenera and showed a clear division between multi-flowered groups (*Polyantha*, *Cochlopetalum*) and single-flowered groups (*Paphiopedilum*, *Sigmatopetalum*). This overall topology closely resembled that of the plastid-derived matK tree (Fig. S1), indicating congruent phylogenetic signals. However, the placement of *Megastaminodium* differed: in *matK*, it was sister to the multi-flowered clade (*Polyantha* + *Cochlopetalum*), while in *PHYC*, its position was unresolved.

A particular source of topological instability involved *P. fairrieanum*, *P. rungsuriyanum*, and *P. hirsutissimum*, which showed variable placements across markers. *P. fairrieanum* and *P. rungsuriyanum* were generally unstable, appearing in polytomies or weakly supported clades, mostly associated with subgenus *Paphiopedilum*. High support was observed in the ITS (Fig. S5) (PP = 0.98), ITS/*XDH* (Fig. S9) (PP = 1.0), *DEF4* (Fig. S4) (PP = 1.0) and combined *LFY/RAD51/ACO/DEF4* analyses (Fig. 1) (PP = 1.0). In contrast, their positions were unresolved in *RAD51* (Fig. S7), *PHYC* (Fig. S8), *ACO* (Fig. S3), and *matK* (Fig. S1). Moderate support was observed in the *ACO/DEF4/PHYC* tree (Fig. 2). By contrast, P. *hirsutissimum* showed a more stable position, consistently recovered within subgenus *Paphiopedilum* with high support across several nuclear markers, including ITS (Fig. S5) (PP = 0.96), *RAD51* (Fig. S7) (PP = 1.0), *XDH* (Fig. S6) (PP = 0.99), *DEF4* (Fig. S4) (PP = 1.0), and all combined nuclear analyses (Figs.1–2 and Fig. S9).

Combined nuclear marker analyses (*ACO/DEF4/PHYC*) (Fig. 2) provided the most robust topologies, consistently supporting all subgenera as monophyletic (PP = 1.0). In these trees, *Megastaminodium* was consistently recovered as sister to the entire clade comprising *Paphiopedilum*, *Sigmatopetalum*, *Cochlopetalum*, and *Polyantha*, confirming its isolated position.

Network analysis using Bayesian *MCMC_GT* showed the same topology of the tree listed in a 95% credible set of topologies (percent = 43,42). In addition, reticulation scenarios were detected. The Maximum a Posteriori (MAP) network, corresponding to the highest posterior probability, was selected as the best-supported evolutionary scenario (log = —38,19,071) are depicted in Fig. 3. Analysis indicates probable ancestral hybridisation in the ancestor of the subgenera *Megastaminodium, Sigmatopetalum*, *Paphiopedilum*, *Polyantha* and *Cochlopetalum*.

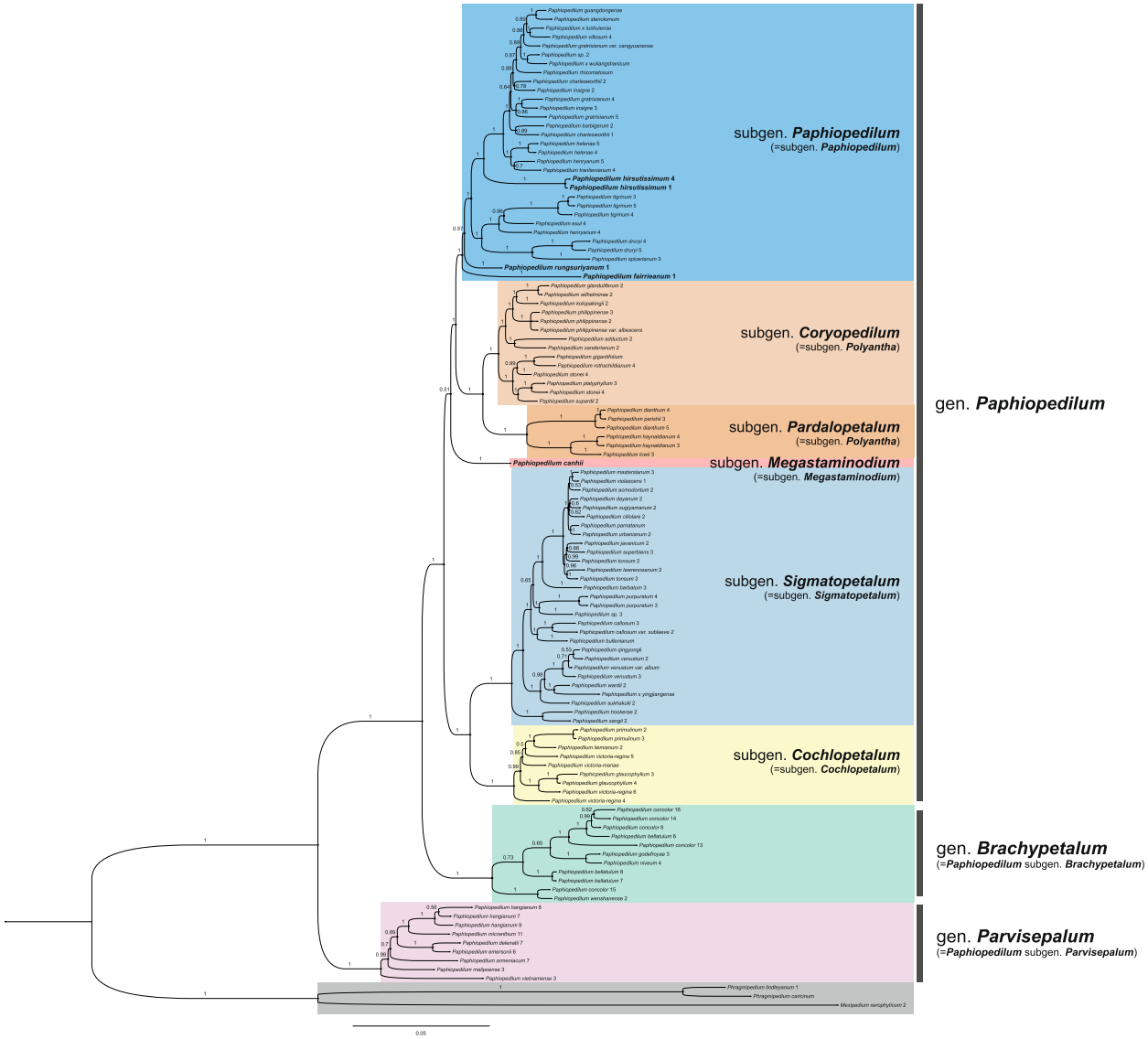

Fig. 1 Bayesian inference phylogeny of the genus *Paphiopedilum* based on a concatenated dataset of the low-copy nuclear genes *ACO* (1-aminocyclopropane-1-carboxylate oxidase), *DEF4* (DEFICIENS-like gene 4), *LFY* (LEAFY), and *RAD51*. Posterior probability (PP) values are indicated above the branches. Taxon annotations on the phylogenetic tree reflect the new systematic framework proposed in this study. For comparison, the classification system according to Braem & Chiron (2003) is shown in parentheses

Biogeographic inference

Paphiopedilum species are distributed across mainland of Southeast Asia and the Sundaic region—an area influenced by repeated Miocene sea-level fluctuations that alternately connected and isolated landmasses, facilitating both vicariance and dispersal. For historical biogeographic reconstruction, the S-DEC model was selected, as it indicated a broader range of ancestral nodes (Fig. 4 A) compared to the BAYAREALIKE + J model (Fig. 4 B). Accordingly, this study focused on the results of the S-DEC reconstruction. The last common ancestor of *Paphiopedilum* (Fig. 4 A) was present in mainland of Southeast Asia. Based on the calibration point used, the common ancestor of the genus existed during the Miocene epoch, approximately 17.5 million years ago. Subsequently, two major evolutionary lineages diverged: one leading to the subgenus *Parvisepalum*, and the other to the ancestor of all remaining subgenera. The time to the most recent common ancestor (tMRCA) of the latter group is estimated at approximately 13 million years ago. This lineage then split into *Brachypetalum* and the common ancestor of the remaining subgenera. The ranges of both early lineages likely overlapped. Divergence-time and biogeographic analyses further indicate that hybridization events occurred between 10 and 8 Ma, likely involving these sympatric lineages, and were followed by dispersal into Sundaland. Ancestral area reconstruction indicates that *Megastaminodium* evolved in continental

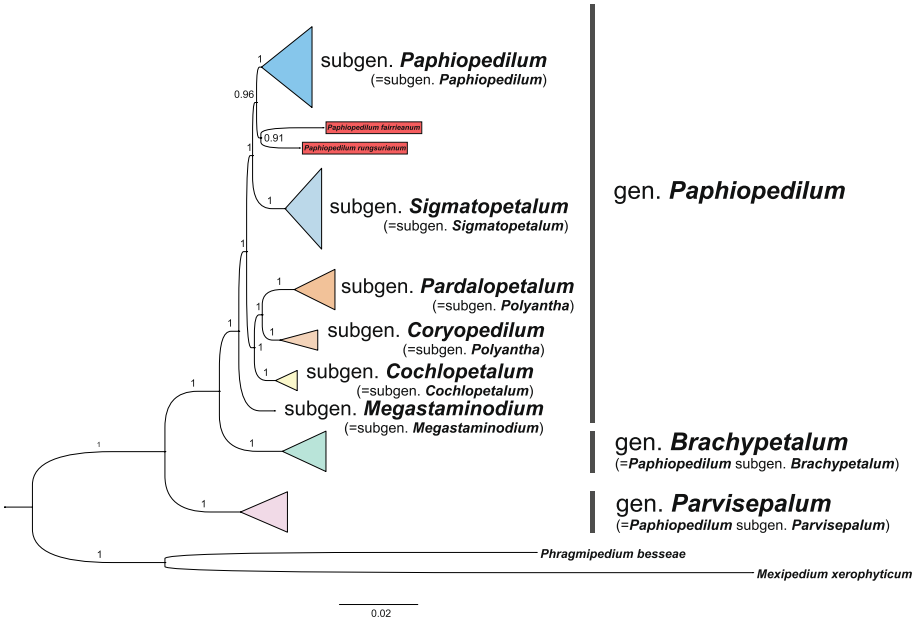

Fig. 2 Bayesian inference phylogeny of the genus *Paphiopedilum* based on a concatenated dataset of the low-copy nuclear genes *PHYC* (phytochrome C), *ACO* (1-aminocyclopropane-1-carboxylate oxidase), and *DEF4* (DEFICIENS-like gene 4). Posterior probability (PP) values are indicated above the branches. Taxon annotations on the phylogenetic tree reflect the new systematic framework proposed in this study. For comparison, the classification system according to Braem & Chiron (2003) is shown in parentheses

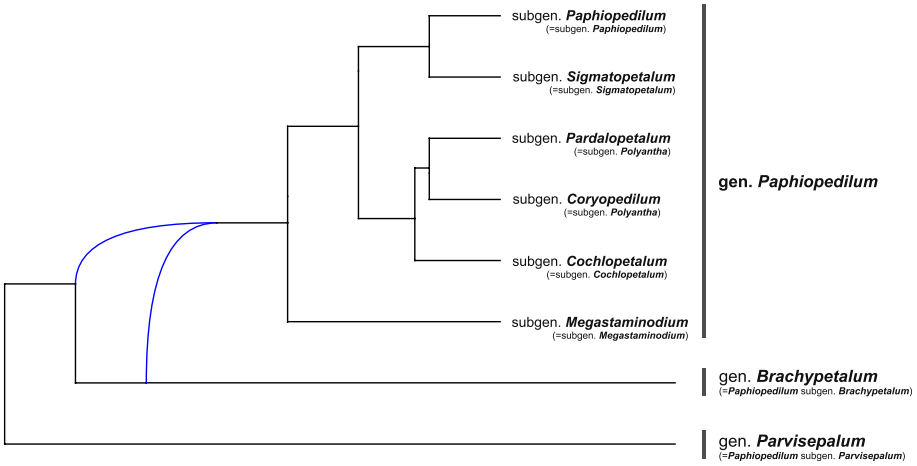

Fig. 3 The Maximum a Posteriori (MAP) network inferred using MCMC_GT, corresponding to the highest posterior probability (log = –38.19071), based on gene tree topologies (nuclear: *ACO*, *DEF4*, *PHYC*, *XDH*, *LFY*, *RAD51*, ITS; and plastid tree), is shown. The blue line indicates a possible hybridization scenario

Southeast Asia, whereas the common ancestor of the other subgenera originated in insular Sundaland (e.g., Borneo). Subsequent marine transgressions likely drove the divergence of the multi-flowered subgenera from the *Paphiopedilum–Sigmatopetalum* clade, with further isolation promoting the diversification of *Sigmatopetalum* and *Cochlopetalum* within the Sundaland islands. Migration back to mainland Southeast Asia is inferred for both the *Sigmatopetalum* and *Polyantha* subgenera.

Taxonomic treatment

An important element of the evolutionary history of the genus *Paphiopedilum* sensu lato includes: (1) a pollinator shift from bumblebees to flies, which influenced its evolutionary trajectory and led to the separation of the ancestor of *Brachypetalum–Paphiopedilum* from *Parvisepalum*; (2) ancestral hybridization events that created mosaic genomes, giving rise to a new evolutionary lineage (*Paphiopedilum* sensu stricto); and (3) adaptive radiation driven by dispersal and geographic isolation in the Sundaland region, resulting in the formation of several evolutionary lineages (subgenera).

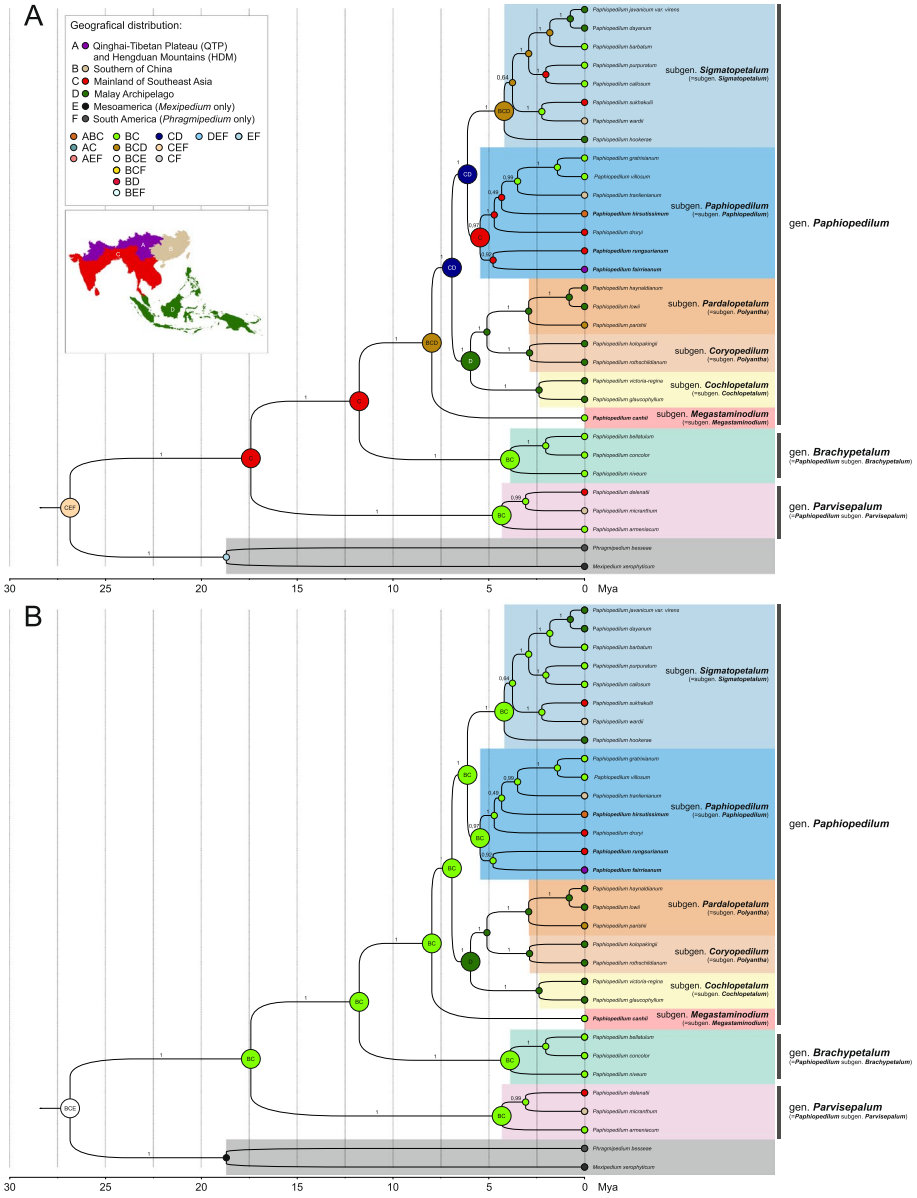

Fig. 4 Ancestral area reconstruction of *Paphiopedilum* based on a concatenated dataset of the low-copy nuclear genes *PHYC* (phytochrome C), *ACO* (1-aminocyclopropane-1-carboxylate oxidase), and *DEF4* (DEFICIENS-like gene 4), along with extant distributions, using (A) the DEC model and (B) BAYAREALIKE + J model in RASP. The map illustrates the coding areas in distinct colors

Below, we propose a new infrageneric classification of *Paphiopedilum* based on combined molecular, morphological, and biogeographical data. Our system differs from all previously proposed classifications (Cribb 1998, Braem & Chiron 2003, Chochai et al. 2012) by recognizing three genera (*Parvisepalum*, *Brachypetalum*, and *Paphiopedilum*), which— in our opinion—constitute monophyletic groups well defined by morphological synapomorphies (Fig. 5). The genus *Paphiopedilum* is further divided into six monophyletic subgenera—*Megastaminodium*, *Paphiopedilum*, *Sigmatopetalum*, *Pardalopetalum*, *Coryopedilum*, *Cochlopetalum*.

We acknowledge concerns regarding potential taxonomic inflation and its impact on conservation and scientific communication. However, precisely delineating evolutionarily meaningful units enhances conservation prioritization by identifying lineages with unique ecological requirements or threats. A well-resolved taxonomy facilitates targeted protection and clearer communication among researchers, conservationists, and policymakers.

Furthermore, our approach balances taxonomic stability with the need to reflect natural evolutionary relationships. Rather than causing confusion, recognizing these genera clarifies slipper orchid evolution and supports effective conservation strategies. This nuanced

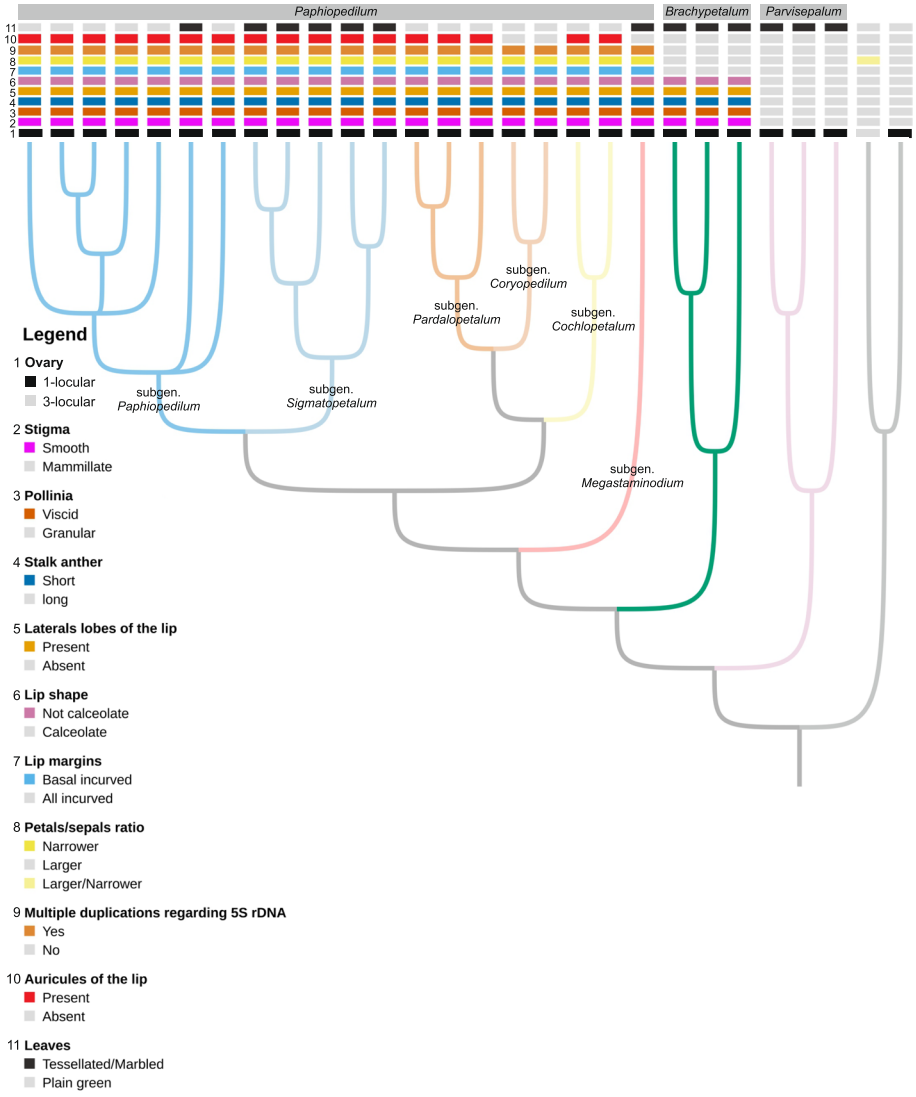

Fig. 5 Bayesian inference phylogeny of the genus *Paphiopedilum* based on a concatenated dataset of the low-copy nuclear genes *PHYC* (phytochrome C), *ACO* (1-aminocyclopropane-1-carboxylate oxidase), and *DEF4* (DEFICIENS-like gene 4), with the overlay of key traits used in the taxonomic circumscription of major clades. Morphological synapomorphies are indicated, as well as the cytogenetic character "multiple duplication of 5S rDNA" (item 9). The tree highlights both genetic relationships and the evolutionary significance of specific morphological features, which are crucial for defining taxonomic boundaries within the genera. Taxon labels reflect the revised systematic framework proposed in this study

framework aligns with modern systematic principles advocating for classifications based on robust phylogenetic evidence, while minimizing unnecessary nomenclatural changes.

Key to the genera:

1* Petals and sepals similar in form, lip with incurved margins … 2
Petals and sepals dissimilar in form, lip apical margins erect or recurved … *Paphiopedilum*
Leaves stiff, sepals relatively small, lip thin-textured, dorsiventrally inflated, stigmatic surface mamillated … *Parvisepalum*

2* Leaves fleshy, not stiff, sepals as large as petals, lip thick-textured, laterally inflated, stigmatic surface smooth … *Brachypetalum*

Genus *Parvisepalum* (Karas. & Saito) Górniak & Szlach., *stat. et comb. nov.*

Basionym: *Paphiopedilum* subg. *Parvisepalum* Karas. & Saito, Bull. Hiroshima Bot. Gard. 5: 31. 1982; Type species: *Paphiopedilum delenatii* Guillaumin ≡ *Paphiopedilum* sect. *Parvisepalum* (Karas. & Saito) P.J. Cribb, Genus Paphiopedilum: 86. 1987.

Genus *Parvisepalum*, embraces several species native mostly to mountain areas of Vietnamese/Chinese borderland. They occupy basal branch of the cladogram

of the genus *Paphiopedilum* s.l.. In accordance with the simple interpretation of phylogenetic tree presented here, this group appears to be the most primitive of the *Paphiopedilum*-related orchids. The species belonging here possess some feature, which seems to present plesiomorphic character state. First of all, it is morphology of the lip. It is usually large, unlobed, thin-textured, dorsiventrally inflated just above the short basal part, with all margins being incurved, obliquely elliptic in shape and obtuse at the top. Similar structure of the lip is observed in all species of *Selenipedium*, *Phragmipedium* subgenera *Micropetalum* (Hallier) Szlach. and *Schluckebieria* Braem and reminds most species of the genus *Cypripedium*. Additionally, staminodium of *Parvisepalum* is subsessile, conspicuous, unlobed, suborbicular, subquadrate, obovate, triangular-ovate, shield-like, what also reminds many species of *Phragmipedium* and *Cypripedium*. It can be speculated that this type of lip and staminodium was inherited from the putative, common ancestor of all lady's slipper orchids. Of course, in some species of *Parvisepalum*-group can be observed modification of staminodial shield (for example *P. micrnathum,* and *P. emersonii*, in which staminodium is not flat, but conduplicate, convex, what reminds for example *C. calceolus*), what can be interpreted as secondary derived character state being adaptation to pollinators. One of the most important evolutionary characters that differs *Parvisepalum* from any other paleotropical species of slipper orchids (*Paphiopedilum*) are glabrous pollinia and the mammillated bottom, fertile surface of stigma. The latter character occurs also in all sister taxa of *Paphiopedilum*, i.e. *Phragmipedium, Mexipedium, Selenipedium* and *Cypripedium* [43]. It seems that this character is synplesiomorphy for the mentioned taxa and was lost in the course of evolution in the common ancestor of all other subgenera of *Paphiopedilum* [19]. Species of *Parvisepalum* are characterized also by numerous secondary character states which evolved as adaptation to specific habitat (karsten limestone hills, montane forest) and pollination systems. To these features belong relatively short and thick, rather stiff and marbled leaves, and large petals which attract, along with large lip, potential pollinators. The only exception in this group are *P. emersonii* and *P. hangianum*, separated by Averyanov and Cribb (2003) in the section *Emersonianum* [44]. Both species share almost plain green, relatively large leaves, and never develop stolons. Despite these differences this section is well embedded in *Parvisepalum*-clade. The results obtained by us indicate that the section *Parvisepalum*, after the separation of the section *Emersonianum* from it, remains a paraphyletic taxon. It is worth noting that species of *Parvisepalum* produce always a single-flowered inflorescence, usually longer than leaves and the sepals are smaller than petals, but similar in form.

Phylogenetic relations between species of the genus *Parvisepalum* depend on the molecular marker used in the analyses and are incongruent. The analysis of ITS (Fig. S5) and *XDH* (Fig. S6) markers, as well as the combined analysis of both markers (Fig. S9), indicates the presence of two groups and two monotypic phylogenetic lineages within the genus *Parvisepalum*. The first group includes the section *Emersonianum*, with *P. micranthum* as its sister taxon. The second group comprises *P. malipoense* and *P. armeniacum*. The monotypic lineages are represented by *P. delenatii* and *P. vietnamense.* The form of the receptive surface of both species is unusual as for *Parvisepalum*. In all species of the subgenus stigma is elliptic. In *P. vietnamense* it is rhombic, and in *P. delenatii* it is strongly elongate basally. In both species the ovary is spotted (also in *P. micranthum*). It is interesting to note that *P. delenatii* is known from southern Vietnam, several hundred kms from the compact geographic occurrence of other species of the group [44]. In general, analyses of other nuclear markers (*LFY, ACO, DEF4, RAD51*) (Figs. S2-S4 and Fig. S7) obtained from the examination of different specimens support the division into these four lineages except the position of *P. delenatii*. The latter is sister to *P. emersonii*. The other conflict position on phylogenetic trees concerns *P. armeniacum*, which, in the case of nuclear markers (ITS, *XDH*) (Figs. S5, S6) is sister to *P. malipoense*, while the *matK* gene (plastid) (Fig. S1) indicates basal position in the *Parvisepalum* for this species altogether with *P. delenatii* and *P. vietnamense* [23].

The results obtained by us indicate that the section *Parvisepalum*, unlike the monophyletic section *Emersonianum*, is a paraphyletic taxon. Therefore, consideration should be given to creating additional sections for groups of species in the *Parvisepalum* or eliminating the division into sections in the genus. We do not support the recognition of sections in *Parvisepalum* and recommend they be merged.

Nomenclatural changes:

Parvisepalum armeniacum (S.C. Chen & F.Y. Liu) Górniak & Szlach., *comb. nov.*

Basionym: *Paphiopedilum armeniacum* S.C. Chen & F.Y. Liu, Acta Bot. Yunnan. 4(2): 163–165, pl. 1, f. 1–4. 1982.

Parvisepalum delenatii (Guillaumin) Górniak & Szlach., *comb. nov.*

Basionym: *Paphiopedilum delenatii* Guillaumin, Bull. Soc. Bot. France 71: 554. 1924.

Parvisepalum emersonii (Koop. & P.J. Cribb) Górniak & Szlach., *comb. nov.*

Basionym: *Paphiopedilum emersonii* Koop. & P.J. Cribb, Orchid Advocate 12(3): 86, f. 1. 1986.

Parvisepalum hiepii (Aver.) Górniak & Szlach., *comb. nov.*

Basionym: *Paphiopedilum hiepii* Aver., Orchids (West Palm Beach) 67(3): 261. 1998.

Parvisepalum jackii (H.H. Hu) Górniak & Szlach., *comb. nov.*

Basionym: *Paphiopedilum jackii* H.H. Hu, Orchideen 46(3): U4. 1995.

Parvisepalum malipoense (S.C. Chen & Z.H. Tsi) Górniak & Szlach., *comb. nov.*

Basionym: *Paphiopedilum malipoense* S.C. Chen & Z.H. Tsi, Acta Phytotax. Sin. 22(2): 119–120, pl. 1. 1984.

Parvisepalum micranthum (Tang & F.T. Wang) Górniak & Szlach., *comb. nov.*

Basionym: *Paphiopedilum micranthum* Tang & F.T. Wang, Acta Phytotax. Sin. 1(1): 56–57. 1951.

Parvisepalum vietnamense (O. Gruss & Perner) Górniak & Szlach., *comb. nov.*

Basionym: *Paphiopedilum vietnamense* O. Gruss & Perner, Orchidee (Hamburg) Beiheft 5: 3. 1999.

Genus *Brachypetalum* (Hallier) Górniak & Szlach., *stat. et comb. nov.*

Basionym: *Paphiopedilum* sect. *Brachypetalum* Hallier, Ann. Jard. Bot. Buitenzorg 14: 34. 1896; Type species: *Paphiopedilum concolor* (Bateman) Pfitzer ≡ *Paphiopedilum* subg. *Brachypetalum* (Hallier) Pfitzer, Pflanzenr. (Heft 12): 55. 1903.

Species of this genus can be found through Indochina, with center of diversification spreading from Chinese/Vietnamese borderland to southern Thailand, on limestone substrates. The South range limit is the border region between Thailand and Malaysia along the Strait of Malacca. In many respects they are similar to *Parvisepalum*-group, both have large petals, incurved lip margins and marbled leaves. In fact, however, in molecular analyses the position of genus *Brachypetalum* is sister to genus *Paphiopedilum.* Their closer affinity is confirmed by morphological features associated with adaptation related to pollinator shift. In contrast to *Parvisepalum* which are pollinated by bumblebees, the genera *Brachypetalum* and *Paphiopedilum* are mainly pollinated by flies (Diptera). These adaptation features include: 1. Smooth surface of the abaxial, fertile part of the stigma; 2. Viscid, sticky pollinia; 3. Short stalk of stigmatic shield and anther. An additional shared characteristic of this genera is the non-calceolate lip. All these features are not present in the genus *Parvisepalum.* In *Brachypetalum* species the lip is laterally compressed, with incurved margins being obliquely triangular, acute, and staminodium is apically 3-lobed or 3-dentate, what can be interpreted as secondary derived states. Relatively often *Brachypetalum*-species produce bi-flowered inflorescences, which are usually shorter than leaves and pendent. The leaves are tessellated, fleshy and not stiff as in the species of *Parvisepalum*. It is interesting to know some similarities between *P. niveum*, representing this subgenus and *P. vietnamense* and *P. delenatii* (both *Parvisepalum*), what can be interpreted as convergency, independent origin of morphological syndromes attracting similar pollinators or evolution of similar pollination mechanism. Molecularly, *P. niveum* is deeply embedded in *Brachypetalum*-clade, and *P. vietnamense* and *P. delenatii* – in *Parvisepalum*.

The other unique features for species of the genus *Brachypetalum* are maroon-spotted flowers, petals and sepals similar in shape and coloration and lip relatively small, laterally inflated with incurved margins.

Relationships within the genus *Brachypetalum* in all analyses are congruent. There are two distinct phylogenetic lineages, both well supported. The first of them includes three species: *P. concolor*, *P. bellatulum*, and their natural hybrid—*P.* x *wenshanense*. The second comprises *P. niveum*, P. *thaianum*, *P. godefroyae*, *P. leucochilum*, and natural hybrid between *P. niveum* and *P. godefroyae—P.* x *ang-thong*. Molecular phylogenetic analyses do not significantly differ from conclusions based on morphological characteristics [15, 17]. They also confirm the assumptions of Braem & Chiron (2003) regarding the origin of both hybrids, *P.* x *wenshanense* and *P.* x *ang-thong*. The only inconsistency concerns the placement of *P. godefroyae* close to *P. niveum*. Both Cribb (1998) and Braem & Chiron (2003) suggested a close relationship between *P. concolor*, *P. bellatulum*, and *P. godefroyae*. The coexistence of *P. niveum* and *P. godefroyae,* along with their natural hybrid, may challenge the speculations [15, 17].

Nomenclatural changes:

Brachypetalum bellatulum (Rchb.f) Górniak & Szlach., *comb. nov.*

Basionym: *Cypripedium bellatulum* Rchb. f., Gard. Chron., ser. 3, 3: 648, f. 99 (pg. 746–7). 1888. ≡ *Paphiopedilum bellatulum* (Rchb.f.) Stein, Orchid.-Buch 456.1892.

Brachypetalum concolor (Bateman) Górniak & Szlach., *comb. nov.*

Basionym: *Cypripedium concolor* Bateman, Bot. Mag. 91: pl. 5513. 1865. ≡ *Paphiopedilum concolor* (Bateman) Pfitzer, Nat. Pflanzenfam. Engler & Prantl 2, 6: 84. 1888.

Brachypetalum godefroyae (God.-Leb.) Górniak & Szlach., *comb. nov.*

Basionym: *Cypripedium godefroyae* God.-Leb., Orchidophile (Argenteuil): 830. 1883. ≡ *Paphiopedilum godefroyae* (God.-Leb.) Stein, Orchid.-Buch: 468.1892.

Brachypetalum leucochilum (Rolfe) Górniak & Szlach., *comb. nov.*

Basionym: *Cypripedium godefroyae* God.-Leb. var. *leucochilum* Rolfe, Orchid. Rev. 2: 145. 1894. ≡ *Paphiopedilum leucochilum* (Rolfe) Fowlie, Orchid Digest 39: 110. 1975.

Brachypetalum niveum (Rchb.f) Górniak & Szlach., *comb. nov.*

Basionym: *Cypripedium niveum* Rchb. f., Gard. Chron.: 1038. 1869. ≡ *Paphiopedilum niveum* (Rchb.f.) Stein, Orchid.-Buch: 478.1892.

Brachypetalum thaianum (Iamwir.) Górniak & Szlach., *comb. nov.*

Basionym: *Paphiopedilum thaianum* Iamwir., Orchid Rev. 114 (1271): 278. 2006.

Genus *Paphiopedilum* Pfitzer

Morph. Stud. Orchideenbl.: 11. 1886; Type species: *Paphiopedilum insigne* (Wall. *ex* Lindl.) Pfitzer.

This taxon is strongly supported by both molecular analyses and morphological evidence. The first one, common for all species classified here is general lip morphology. Despite species of *Parvisepalum* and *Brachypetalum*, the lip of *Paphiopedilum* is rather stiff, bi-partite and comprises of basal, narrow, tubular part, which margins—so called lateral lobes—are incurved and often meeting beneath staminodium.The apical part of the lip is much swollen with apical margin remaining straight or slightly recurved. Its lateral margins form so-called auricles, which are well-developed and incurved. The second character is a possession of relatively narrow petals, surely much narrower than sepals and different in form. At the cytological level, the genus *Paphiopedilum* is also characterized by multiple duplications of 5S rDNA, a feature not observed in the two other genera [20, 45]. These duplications may reflect post-hybridization genomic instability, including segmental duplications and deregulation of repeat sequence maintenance mechanisms. The evolutionary origin of *Paphiopedilum* sensu stricto can be best understood in light of the complex interplay between hybridization, geographic range shifts, and environmental dynamics. Rather than relying solely on morphological traits, our framework incorporates evidence of ancient gene flow and regional radiations to explain the emergence of well-supported subgeneric clades. These processes, discussed earlier, provide the foundation for our revised taxonomic treatment. Due to the presence of well-defined morphological synapomorphies and strong phylogenetic support, five major lineages within the genus *Paphiopedilum* (corresponding to sections recognized by Cribb, 1998) have been elevated to the rank of subgenera. We have also included the monotypic subgenus *Megastaminodium*, established by Braem and Gruss (2011) for *Paphiopedilum canhii* [22]. Similarly, Averyanov placed it in the *Pygmea* section within the subgenus *Paphiopedilum* sensu Cribb [44].

Key to the subgenera

Inflorescence usually single-flowered … 2
1* Inflorescence multi-flowered … 4
Leaves stiff, marbled, staminodium shield-like, very large, as long as the lower part of the lip … *Megastaminodium*
2* Leaves thin-textured, if stiff than plain green, staminodium smaller. Distinctly shorter than the lower part of the lip … 3
Leaves mottled, petals often warty, staminodium crescent-like, 3-lobed at apex … *Sigmatopetalum*
3* Leaves usually plain green, petals not warty, staminodium usually with central umbo … *Paphiopedilum*
Flowers opening successively … *Cochlopetalum*
4* Flowers opening simultaneously … 5
Petals' base perpendicular to the dorsal sepal, horn- or knob-like projection at the base of usually glabrous staminodium present … *Pardalopetalum*
5* Petals' base parallel to the dorsal sepal, long, geniculate filament of staminodial shield present, margins of staminodium usually long pubescent … *Coryopedilum*

Subgenus *Megastaminodium* Braem & O. Gruss

Orchid Digest 75(3): 164. 2011; Type species: *P. canhii* Aver. & O.Gruss = *Paphiopedilum* sect. *Megastaminodium* (Braem & O. Gruss) Y.I Lee, M.C.Chung, Sydara, Souliya & Luang Aphay, Bot. Stud. (Taipei) 58–16: 9. 2017. = *Paphiopedilum* sect. *Pygmaea* Aver., Planet. Orchid. 24(4): 20. 2011.

A detailed discussion concerning the taxonomic position of *Paphiopedilum canhii* was presented by Górniak et al. (2014). Lee et al. (2017) found *P. canhii* to be sister to subgenus *Paphiopedilum* based on combined plastid and nuclear data [20]; however, this placement was supported exclusively by the *DEF4* and *RAD51* markers. Importantly, this result was not confirmed by other markers used in their study [20], nor was it supported by our own analyses, including updated datasets for *DEF4* (Fig. S4) and *RAD51* (Fig. S7). In our phylogenetic reconstructions, *P. canhii* consistently occupies a basal position relative to all other subgenera of *Paphiopedilum*, indicating that it represents an early-diverging evolutionary lineage within the genus. The isolated position of *P. canhii* in our analyses, along with its unique morphological traits—such as a disproportionately large staminodium, distinctive lip morphology, absence of warts on the lip and petals, and marbled, relatively stiff leaves with species-specific adaxial epidermal cells—supports its recognition as a separate subgenus. This placement is further supported by tree topologies obtained using PhyloNet (Fig. 3).

Subgenus *Paphiopedilum*

Our concept of subgenus *Paphiopedilum* follows Karasawa & Saito (1982) and Braem & Chiron (2003) [13,

17]. The species of this subgenus are distributed across mainland Southeast Asia. Molecular analyses confirm the distinctness of these groups. Species of sect. *Paphiopedilum* (sensu Karasawa & Saito) are morphologically uniform and characterized by an obovate to obcordate staminodium with a central umbo, which we consider a synapomorphy.

The subdivision of this clade into subunits correlates with geography and partially with species complexes proposed by Braem & Chiron (2003) [17], including a *'P. villosum* complex'that also comprises *P. insigne*. The basal clades of the subgenus contain morphologically divergent species, such as *P. fairrieanum* (sect. *Ceratopetalum*), *P. hirsutissimum* (sect. *Stictopetalum*), and *P. druryi* and *P. spicerianum* (sect. *Thiopetalum*). All of these lack a central umbo on the staminodial shield. With the exception of *P. hirsutissimum*, they occur at the periphery of the subgenus's geographic range.

The phylogenetic positions of *P. fairrieanum* and *P. hirsutissimum* have been unresolved in previous studies. Our single-marker analyses (except *DEF4*) (Fig. S4) also do not resolve their placement. However, combined analyses (ITS/*XDH* and *ACO/DEF4/LFY/RAD51*) (Fig. S9 and Fig.1) strongly support (PP = 1.0) their inclusion in subgenus *Paphiopedilum*. The taxonomic position of *P. rungsuriyanum* has likewise been debated. Although it was assigned to the monotypic section *Laosianum* by Lee et al. (2017), based in part on a presumed relationship with *P. canhii*, our data do not support this interpretation (see Subgenus *Megastaminodium*). All combined analyses place *P. rungsuriyanum* firmly within subgenus *Paphiopedilum*. Therefore, we do not recognize it as a separate subgenus. However, we propose retaining section *Laosianum* within subgenus *Paphiopedilum sensu* Karasawa & Saito (1982) [13]. It remains possible that *P. fairrieanum* and *P. rungsuriyanum* have hybrid origins. For example, the staminodial shield of *P. fairrieanum* resembles that of some *Barbata* species (e.g., *P. appletonianum*), while another possibility is that these species retain plesiomorphic traits from a common ancestor of *Paphiopedilum* and *Sigmatopetalum.*

Subgenus *Sigmatopetalum* Hallier f. *ex* Karas. & Saito

Bull. Hiroshima Bot. Gard. 5: 42. 1982; Type species: *Paphiopedilum venustum* (Wall. *ex* Sims) Pfitzer.

Subgenus *Sigmatopetalum* is the most widespread in the genus. Its range extends from eastern Himalaya through southern China, Indochina, the Philippines, Indonesia to Bougainville Islands. There are several features which can be treated as synapomorphies of this group: tessellated leaves, more or less crescent or semilunate staminodium, pubescent on the outer surface, usually warted lip lateral lobes and petals, pubescent on margins and warted in most species. All characters are not found elsewhere in the genus (see *P. dodyanum* of the subgenus *Cochlopetalum*), making this group well defined and separated from other *Paphiopedilum* groups. We suppose that wasting of any warted callosities in the lip lateral lobes and petals noted in some species, like *P. sangii, P. bougainvilleanum, P. violascens* and *P. wentworthianum*, happened during evolution, probably because of adaptation to the pollinators.

The subdivision into smaller units was carried out by Karasawa and Saito (1982) [13]. However, in our discussion, we will refer to the system proposed by Braem (1998) [16] and Braem & Chiron (2003) [17], who developed their system based on the Karasawa and Saito (1982) system, incorporating new species described over the next 20 years. The authors identified six sections, dividing two of them into a further two subsections. Although this detailed division was not accepted by other authors (Averyanov et al. 2003) [44]. In our discussion, we will attempt to relate to this division in the context of molecular analyses. Some groupings within sections are monophyletic, whereas sections as a whole are polyphyletic. This demonstrates the independent evolution of the traits taken into account in creating the intrageneric classification of subgenus *Sigmatopetalum* by Karasawa and Saito (1982) and Braem & Chiron (2003) [13, 17].

The best phylogenetic resolution was achieved based on the combined analysis of *RAD51*/*LFY*/*ACO*/*DEF4* markers (Fig. 1). The first node splits the subgenus clade into two lineages. One of them consists of *P. sangii* and *P. hookerae*. Both species were placed by Braem & Chiron (2003) in the *Macronodium* subsection of the *Spathopetalum* section [17]. Cribb (1998) also points to their close relationship [15]. The common features of both species are a large circular staminode, a chromosome number of 2n = 28, intense venation of the pouch, and coloring and shape of the petals. Based on their position on the trees and chromosome number, we can assume that these two species are basal for the subgenus *Sigmatopetalum*. However, molecular studies do not confirm a close relationship between the subsection *Macronodium* and the *Spathopetalum* subsection, which includes *P. appletonianum*, *P. bullenianum*, and *P. cerveranum*. Mentioned species form a clade in the ITS marker analysis (Fig. S5) but all together with remaining species of the subgenus compose second lineage. Within this lineage two larger clades can be distinguished (*RAD51*/*LFY*/*ACO*/*DEF4*) (Fig. 1). The first clade includes species from nominal section (monotypic *P. venustum*) and two species from section *Planipetalum* (*P. wardii* and *P. sukhakulii*), mainly found in the continental part of Southeast Asia.

The second clade includes species found in the Philippines, Borneo, Java and Solomon Islands. According to the system of Braem & Chiron (2003), the species in this clade come from the sections *Barbata, Blepharopetalum*,

Punctatum, *Planipetalum* (only *P. purpuratum*) and subsection *Spathopetalum* (*P. bullenanium*) [17]. Based on the analysis of all markers, it is challenging to indicate the relationships between these species. Certain species form strongly supported groupings classified in subsections from section *Barbata*. But, like sections also subsections are polyphyletic. Based on current molecular data, it seems reasonable to combine species from all these sections into a single section *Barbata*. Another challenge is to diagnose common morphological characters and/or anatomical features for section *Barbata s.l.*

Multiflowered subgenera

The multiflowered *Paphiopedilum* species (subgenera *Polyantha* and *Cochlopetalum*) form a monophyletic group, based on *matK* (plastid) (Fig. S1) and nuclear *ACO*/*DEF4*/*PHYC* analyses (Fig. 2). Despite the synapomorphies indicated, there are many differences between multi-flowered subgenera, in this number the sequence of the flower development – in the representatives of the subgenus *Cochlopetalum* they are successively opened, and in *Polyantha* – simultaneously opened. In our opinion, it is possible to formulate a set of advanced and unique features for each of these subgenera. The subgenus *Polyantha* was divided into three sections (*Polyantha*, *Mystropetalum,* and *Mastigopetalum*) by Karasawa and Saito (1982) and Braem & Chiron (2003) [13, 17]. Each of these sections is monophyletic and well supported in most of our analysis. The sections *Polyantha p.p.* and *Mystropetalum* form a monophyletic group and together they form a clade. Cribb (1998) combined species from both sections into one section of *Pardalopetalum* [15]. On the other hand, species from the *Mastigopetalum* section were placed in a separate section named *Coryopedilum*. We follow Cribb on his decision and additionally raise both section to subgenus rank.

Subgenus *Pardalopetalum* (Hallier & Pfitzer) Górniak & Szlach., *stat. et comb. nov.*

Basionym: *Paphiopedilum* sect. *Pardalopetalum* Hallier & Pfitzer, Pflanzenr. 50 (Heft 12): 66. 1903; Type species (Cribb 1989: 172): *Paphiopedilum lowii* (Lindl.) Stein.

Species belonging to this subgenus can be characterized by at least two synapomorphies. They are horn- or knob-like projection at the base of usually glabrous staminodium, and oblique connection between petals and ovary (petals' bases are perpendicular to the base of the dorsal sepal). Section *Polyantha* (*P. haynaldianum* and *P. lowii*) is characterized by the presence of lateral ears on the main lobe of the lip and spoon-shaped petals with purple spots. The latter species was found throughout the Malay Peninsula, Sumatra, Java, Borneo and Sulawesi. On the other hand, *P. haynaldianum* is restricted only to the Philippines. The second section (*Mystropetalum)* comprises species (*P. parishii* and *P. dianthum*) whose petals are strongly twisted and do not expand towards the apex. Their coverage includes the continental part of Southeast Asia.

Subgenus *Coryopedilum* (Pfitzer) Górniak & Szlach., *stat. et comb. nov.*

Basionym: *Paphiopedilum* sect. *Coryopedilum* Pfitzer, Pflanzenr. 50(Heft 12): 59. 1903; Type species (Cribb 1998: 124): *Paphiopedilum glanduliferum* (Blume) Stein.

To this subgenus belongs species with greatly diversified staminodium, what is probably adaptation to the various syndromes of pollination/pollinators. Generally, we can characterize those species by having long filament and staminodial shield geniculately connected with it. The margins of staminodium are usually long pubescent. Specific staminodia correlated with morphology of the flower parts make clear limits between species. The results of the molecular analyses we obtained allow us to verify previous assumptions regarding the relationships in this group. Braem & Chiron (2003) distinguished within the section *Mastigopetalum* (*Coryopedilum* sensu Cribb 1998) several complexes [17].

Low-copy nuclear genes, ITS (Fig. S5), and plastid (Fig. S1) trees are incongruent with each other. In the ITS phylogeny, the section *Coryopedilum* does not form a monophyletic group; however, the results also do not support its polyphyly. In contrast, analyses based on nuclear low-copy markers (*LFY, ACO, DEF4, RAD51*) (Figs. S2-S4 and Fig. S7) and plastid genome data support the monophyly of this section. The differences in topology concern the relationships between clades/species complexes. The phylogenetic tree based on the analysis of ITS sequences is the most diverse in terms of species. Therefore, initially, we will focus on the topology of this tree. In the section *Mastigopetalum*, groupings corresponding to complexes distinguished by Braem & Chiron (2003) can be observed, some of which also align with geographical distribution. The species cluster from Mindanao Island (*Paphiopedilum adductum* complex) is strongly supported (PP = 1.0). Moderately supported (PP = 0.97) are the species *P. philippinense* and *P. sanderianum*. Another clade consists of species belonging to the *P. praestans* complex from New Guinea. An independent lineage in all the groups is formed by *P. intaniae*. All the species form an unsupported clade (PP = 0.93). Another low supported clade (PP = 0.95) includes species from Borneo Island: *P. stonei*, *P. kolopakingii*, *P. platyphyllum*. These species have a distinctive large staminode with hairs at its base and edges. Independently from the rest, another species from Borneo, *P. rothschildianum*, occurs. *P. rothschildianum* deviates from this staminode pattern, having a narrowing staminode towards the apex.

In the combined *RAD51/LFY/ACO/DEF4* phylogenetic tree (Fig. 1), two major clades are clearly resolved (PP = 1.0). The first clade comprises the majority of species from Borneo Island (excluding *Paphiopedilum kolopakingii* and *P. sanderianum*), and also includes *P. gigantifolium* from Sulawesi. The second clade consists of two species complexes: the *P. adductum* complex from Mindanao, together with its sister species *P. sanderianum*, and the *P. praestans* complex from New Guinea, which is sister to *P. kolopakingii*. Additionally, *P. philippinense* is recovered as sister to this latter subclade.

The primary difference between the ITS tree (Fig. S5) and the combined analysis (Fig. 1) concerns the placement of *P. kolopakingii*. Notably, its position in the combined nuclear dataset is corroborated by the plastid phylogeny. Both nuclear datasets support a close evolutionary relationship among species occurring on Mindanao (*P. adductum* complex), the *P. philippinense* complex, and those from New Guinea (*P. praestans* complex). These phylogenetic patterns are congruent with their respective geographic distributions.

In the plastid marker analysis (Fig. S1), species from New Guinea (*P. praestans* complex) form a clade together with several Bornean species, including *P. platyphyllum*, *P. stonei*, and *P. kolopakingii*. The second major clade comprises species from Mindanao (*Paphiopedilum adductum* complex), along with additional species from Borneo (*P. rothschildianum*, *P. sanderianum*, and *P. ooii*), and members of the *P. philippinense* complex. Morphological characteristics of the staminodium support this division. The evolutionary history of *Coryopedilum* appears to be highly complex, likely shaped by extensive hybridization events that contributed to the origin of many species within the group (Bornean species). Further molecular studies incorporating multiple low-copy nuclear genes will be essential for uncovering the underlying evolutionary processes and resolving remaining phylogenetic ambiguities.

Subgenus *Cochlopetalum* (Hallier f. *ex* Pfitzer) Karas. & Saito

Bull. Hiroshima Bot. Gard. 5: 55. 1982; Type species: *Paphiopedilum victoria-regina* (Sander) M.W. Wood = *Paphiopedilum* sect. *Cochlopetalum* Hallier *ex* Pfitzer, Pflanzenr. 50(Heft 12): 68. 1903.

The subgenus *Cochlopetalum* is restricted in distribution to Indonesian islands – Java and Sumatra. The group is relatively uniform. Leaves are relatively short and wide, thin and stiff, ciliate along margins towards base. They are either plain green, often with darker venation, or purple spotted beneath or above. The only exception is recently described *Paphiopedilum dodyanum* which leaves are tessellated like in the most species of the subgenus *Sigmatopetalum*. All species classified here are characterized by successively opened flowers and inflorescence being cochlea-shaped in the early stage of development. The staminodial shield is sessile, relatively large, covering from above entirely stigmatic surface and anthers, entire, somewhat convex, pubescent or ciliate in the basal part. In this respect the species of the subgenus are very similar to each other. There is no projection on the staminodial shield except *P. victoria-reginae*, which has basal, rudimentary, digitate appendix. Petals are twisted in the apical half, undulate and variously pubescent along margins. The lip basal part is distinctly shorter than in other members of the nominal subgenus. All species of this group are easily recognizable from other *Paphiopedilum*, but limits between species of this section are not perspicuous and sometimes were questioned by various authors.

Conclusion

Our findings offer a substantially revised classification of the *Paphiopedilum* complex, informed by a synthesis of molecular, morphological, and biogeographic evidence. The robust phylogenetic framework confirms the monophyly of the major lineages and clarifies relationships that have remained uncertain for decades.

Crucially, our analyses reveal that an ancestral hybridization event has shaped the diversification of the genus—challenging traditional morphological groupings and supporting the need for a new taxonomic structure. We propose recognizing *Parvisepalum*, *Brachypetalum*, and *Paphiopedilum* as distinct genera, with the latter comprising six well-supported subgenera defined by clear synapomorphies.

This refined classification not only aligns with evolutionary history but also establishes a more stable and predictive system for ongoing work in orchid biology. It provides an essential reference point for future studies on speciation, trait evolution, and conservation strategies within this ecologically and horticulturally important group.

Abbreviations

ACO	1-Aminocyclopropane-1-carboxylate oxidase
AICc	Corrected Akaike Information Criterion
BEAST	Bayesian Evolutionary Analysis Sampling Trees
BLAST	Basic Local Alignment Search Tool
DEF4	DEFICIENS-like gene 4
ICBN	International Code of Botanical Nomenclature
ILS	Incomplete Lineage Sorting
ITS	Internal Transcribed Spacer
J	Jump dispersal / Founder-event speciation
LFY	LEAFY
LRT	Likelihood Ratio Test
MAP	The Maximum a Posteriori
matK	Maturase K
MCMC	Markov Chain Monte Carlo
MCMC_GT	Markov Chain Monte Carlo using Gene Trees
MSNC	Multispecies Network Coalescent Framework
Mya	Million years ago
PHYC	Phytochrome C

PP	Posterior Probabilities
QTP	Qinghai-Tibetan Plateau
RASP	Reconstruct Ancestral State in Phylogenies
S-DEC	Statistical Dispersal-Extinction-Cladogenesis
tMRCA	Time to the Most Recent Common Ancestor
XDH	Xanthine dehydrogenase

Supplementary Information

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Supplementary Material 1.

Supplementary Material 2.

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Authors' contributions

Conceptualization, M.G. and D.L.S.; methodology, M.G., N.O. and D.L.S.; validation, M.G. and D.L.S.; formal analysis, M.G.; investigation, M.G. and D.L.S.; writing—original draft preparation, M.G. and D.L.S.; writing—review and editing, M.G.; visualization, NO.; supervision, D.L.S. and M.G.; funding acquisition, M.G. All authors have read and agreed to the published version of the manuscript.

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Data availability

Sequence data that support the findings of this study have been deposited in GenBank under the following accession numbers: ACO: PV684576–PV684582, DEF4: PV684583–PV684586, RAD51: PV684587–PV684590, LFY: PV684591–PV684593, PHYC: PV693818–PV693830, XDH: PV704403–PV704478, PV941805–PV941806, matK: PV704479–PV704579, PV941798–PV941804, ITS: PV704793–PV704897, PV938549–PV938557.
The data matrices underlying this study have been deposited in Figshare and are accessible via https://doi.org/10.6084/m9.figshare.28981424.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

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Competing interests

The authors declare no competing interests.

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