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.

Key words: Ancestral character state reconstruction, protandry, environmental sex determination, multilocus phylogeny, reproduction

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 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 constraints during an individual's life history (Schlessman, 1988; Korpelainen, 1998). Thus, ESD plants are able to produce staminate, pistillate or even bisexual 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 & 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., *Grobysa* 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 & 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.

Material and methods

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 *ycf1*, as well as the 'cp' *trnS-trnG* 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' *trnS-trnG* 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., 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 conflicting 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.

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 lock 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 Catsetinae 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 & 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 Catsetinae 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 $\rightarrow$ unisexual) and *vice versa* (q_{20}), plus reversals from the states 1 to 0 (q_{10} : bisexual, protandrous flowers $\rightarrow$ 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 BayesFactors (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).

Results

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 Material and Methods section), MP, ML and Bayesian trees of the ‘n’ and ‘cp’ data partitions recovered very similar, non-conflicting phylogenies (data not shown).

Figure 2 shows the Bayesian majority-rule consensus tree as inferred from the concatenated alignment, with many nodes exhibiting high if not maximal support. Irrespective 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.

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 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’ (Tab. 2), the asymmetrical rate model (ARD) was favoured against equal rates evolutionary models (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 Figure 3, with ancestral character states of ESD (left) and protandry (right) independently inferred under the 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 Tab. 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 (95 LBS, .65BPP). Such ancestral conditions were present also in the LCA of *Grobysa* (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*+*Cynoches*+*Mormodes*), the protandrous condition was retained, but ESD was absent. At node L, the LCA of *Cynoches* and *Mormodes* had protandrous flowers probably coupled with absence of ESD. In the respective LCAs of *Catasetum* (Node F) and *Cynoches* (Node I) as only lineages consistently exhibiting ESD, unisexual flowers were present, and the derived status of ESD was thus confirmed.

Figure 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 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).

Discussion

Previous phylogenetic studies of Catasetinae (Batista et al., 2014; Chase and Phippen, 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*, *Cynoches* 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, 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 state 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 Catsetinae (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 Catsetinae's LCA inferred under alternative approaches. Additionally, it supports the single gain of protandry in the LCA of core Catsetinae (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 Tab. 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 Methods). 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 Catasetina 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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Figure Legends

Figure 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 *Cynoches guttulatum* Schltr. E) Protandrous flower of *Mormodes maculata* (Klotzsch) L.O. Williams in staminate (left) and pistillate (right) phase. Photos: G. Salazar & O. Pérez.

Figure 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 thicker 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.

Figure 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.

Figure 4. Posterior distribution of rate coefficients and their respective mean and standard deviation obtained from 30000 observations sampled from 50 million iterations of a RJ-MCMC 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.

Tables

Table 1. Alignment characterisation

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).

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.

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.

Figure 1

Figure 2

Figure 3

Figure 4

Table 1

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

Table 2

			Models		
Trait	States		Equal rates (ER)	Asymmetrical rates (ARD)	LRT
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	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

Table 4

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