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RESEARCH ARTICLE

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Ecological and phylogenetic constraints determine the stage of anthetic ovule development in orchids

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Abstract

Premise: Unlike most flowering plants, orchid flowers have under-developed ovules that complete development only after pollination. Classical studies reported variation in the stage in which ovule development is arrested, but the extent of this variation and its evolutionary and ecological significance are unclear.

Methods: Here, we used light microscopy to observe ovule development at anthesis for 39 species not previously studied and surveyed the literature gaining information on 94 orchid species. Tropical and temperate members of all five orchid subfamilies as well as species with contrasting pollination strategies (rewarding versus deceptive) and life forms (epiphytic versus terrestrial) were represented. We analyzed the data using statistical comparisons and a phylogenetic generalized least square (PGLS) analysis.

Results: Apostasioideae, the sister to the rest of the orchids, have mature ovules similar to other Asparagales, while under-differentiated ovules are present in the other subfamilies. Ovule developmental stages showed high variation even among closely related groups. Ovules were more developed in terrestrial than in epiphytic, in temperate than in tropical, and in rewarding than in deceptive pollination orchid species. This latter comparison was also significant in the PGLS analysis.

Conclusions: These results suggest that ovule developmental stage in orchids can be shaped by ecological factors, such as seasonality and pollination strategy, and can be selected for optimizing female reproductive investment.

KEYWORDS

flowering time, life form, megagametophyte, Orchidaceae, ovule integuments, pollination strategy, reproductive investment

The ovule is a fundamental player in the crucial process of sexual reproduction in plants. This organ is one of the main evolutionary novelties of spermatophytes and is likely the basis of their extraordinary evolutionary success. In the gymnosperms, ovules and seeds have a number of functions such as pollen capture, propagule protection, and dispersal, resulting in a complex organization and structure (Smith, 1964). In angiosperms, the functional constraints acting on gymnosperm ovules development are relaxed by the evolution of a new structure, the carpel, that oversees pollination, seed protection, and dispersal (Leslie and Boyce, 2012). Such transfer of functions from ovules to carpels may have allowed angiosperms to respond readily to selective pressures, hence favoring more efficient ovule developmental patterns with respect to earlier spermatophytes (Leslie and Boyce, 2012). Accordingly, before fertilization, ovules are generally less developed in the angiosperms than in the gymnosperms. This anthetic ovule reduction (i.e., occurring when flower elements are fully differentiated but fertilization has not occurred) allows for a smaller initial investment in the angiosperm ovule (Lord and Westoby, 2012) with maternal resources allocated to developing seeds only after fertilization.

In the ovule, the two generations of the embryophyte life cycle coexist. In this organ, the diploid maternal sporophytic generation (the inner and outer integuments and the nucellus) embeds and sustains haploid gametophytic generation (the embryo sac; Endress, 2011) with the ovule sporophytic maternal tissues exerting control over the developing haploid generation (Bencivenga et al., 2011).

A master role for the sporophytic on the gametophytic tissue is confirmed by the fact that many mutants affecting the development of the sporophytic tissues of the ovule disrupt female gametophyte development. These mutations include many genes, expressed in the sporophytic tissue and not expressed in the haploid cell line, that impair the maturation of megagametophyte. *AINTEGUMENTA* (*ANT*), *BELL1*, *SEEDSTICK* (STK) and other genes (reviewed by Pinto et al., 2019; see also Dirks-Mulder et al., 2019 and references therein) encode transcription factors involved in sporophytic integument development. In *ant* and *bell1* mutants, ovules have reduced or absent integuments, and embryo sacs are arrested at the 1-nucleate stage, confirming the importance of the sporophytic maternal tissues to promote and control megagametophyte formation and the existence of continuous crosstalk between the two generations. At the same time, there is increasing evidence that hormones such as ethylene act as messengers in this sporophyte–megagametophyte crosstalk. When ACC (1-aminocyclopropane-1-carboxylate) oxidase, a key enzyme for ethylene metabolism, is silenced or inhibited, ovule development is arrested and megasporocytes are unable to start or complete their formation (Bencivenga et al., 2011).

In most angiosperms, in a synchronized process with the development of sporophytic integuments, the megagametophyte develops from the functional megaspore through three syncytial divisions forming an 8-nucleate embryo sac; after cellularization, typically seven cells are formed, two synergids and an egg cell at the micropylar pole, the binucleate central cell in the middle, and three antipodals at the chalazal pole (i.e., the *Polygonum* type ovule development; see Figure 1) (Yadegari and Drews, 2004). Ovule structure and the embryo sac are generally completely developed when pollen grains germinate on the stigma. As a result, fertilization normally occurs shortly after pollination (Mòl et al., 1994; Christensen et al., 1997; Faure et al., 2002; Wu et al., 2011). Although the resource investment in ovule development before pollination is minimal (seven cells) in angiosperms, in some cases, megagametophyte development is either absent or incomplete before pollination.

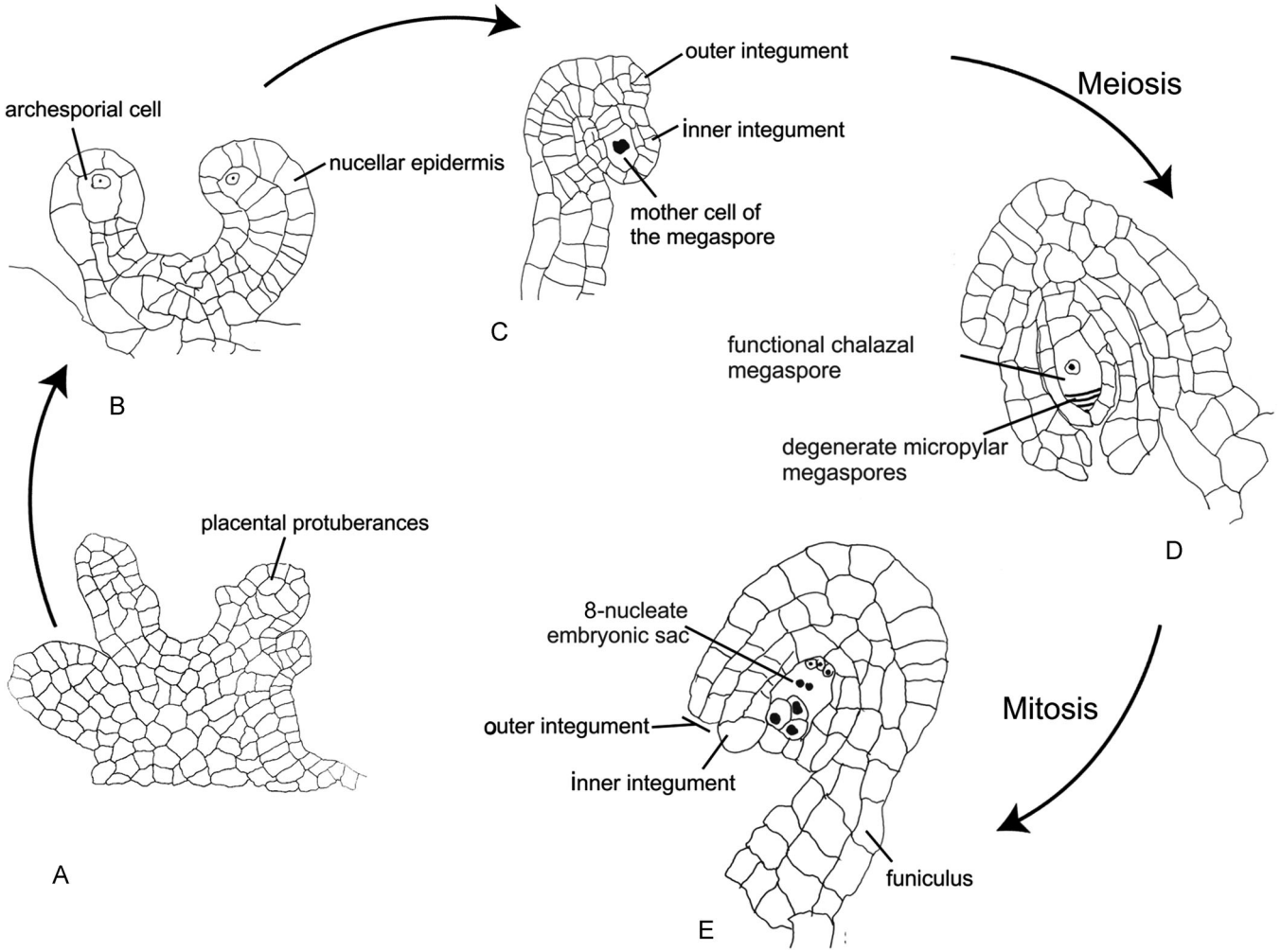

FIGURE 1 Schematic representation of ovule development in flowering plants. (A) Protuberances start to form on the placenta. (B) Formation of ovule primordia with the archesporial cell. (C) The archesporial cell becomes the mother cell of the megaspore, and integuments start to develop. (D) Formation of the megaspore after meiosis. (E) Mature ovule with 8-nucleate embryo sac

Ovule under-differentiation at anthesis has been reported for a few species of Fagales, Rosales, *Nicotiana* (Pimienta and Polito, 1983; O'Neill, 1997; Sogo and Tobe, 2006a, b; Liu et al., 2014; Brito et al., 2015) and, notably, most orchids (Arditti, 1992; Zhang and O'Neill, 1993; Yeung and Law, 1997). For these plants, pollination is important to trigger or regulate embryo sac development and ovule maturation that subsequently allows fertilization.

Orchids offer several examples of ovules arrested at different developmental stages before pollination. For instance, in several genera, at anthesis, ovule primordia are arrested at the pre-meiotic stage: in *Cattleya*, *Phalaenopsis*, *Dendrobium*, and *Oncidium*, pollination triggers initiation and development of ovules (Duncan and Curtis, 1943; Israel and Sagawa, 1964; Zhang and O'Neill, 1993; Mayer et al., 2011) that are not differentiated in the unfertilized ovaries (e.g., Figure 1A); in *Cypripedium* and *Paphiopedilum*, ovule primordia are present but arrested before pollination (Duncan and Curtis, 1942; e.g., Figure 1B, C). In other genera such as *Epipactis*, ovules are fully developed (post-meiotic stage) before pollination (Fredrikson, 1992; e.g., Figure 1D, E). Such variability in megagametophyte developmental stages at anthesis in orchids thus offers the unique opportunity of testing the evolutionary and ecological role of this unusual ovule developmental timing.

The reduction of anthetic ovule development can represent an important resource allocation strategy that can assume a higher significance considering the extremely elevated number of ovules produced in orchid ovaries. Thus, under-developed ovules can be advantageous in circumstances of low probability of pollination as often happens in deceptively pollinated orchids (i.e., species that offer no reward to their pollinators) (Tremblay et al., 2005). A complementary hypothesis (Swamy, 1943) suggests that variation in the developmental stage of the anthetic orchid ovules can be related to the environment in which they grow or to their life form (terrestrial or epiphytic). Highly seasonal environments, where plants grow and reproduce during short periods of time, may favor more developed ovules at anthesis, as a reproductive assurance (Barone-Lumaga et al., 2019). At the same time, orchids with different life forms may slow their development either by disappearing underground (terrestrials) or shedding leaves (epiphytes).

Here, by collecting new evidence and collating previous literature, we evaluated whether ecological or phylogenetical constraints may play a role in shaping the stages of ovule development at anthesis in orchids. Specifically, we tested the hypothesis that ovules at anthesis are less developed in (1) orchids that do not experience strong seasonality (as many tropical species), which have more time to complete ovule development after pollination, and (2) orchids experiencing low fruiting success (deceptively pollinated species), which are exposed to high risk of failure of reproductive investment. In detail, we asked the following questions: (1) To what extent does ovule development vary across orchid lineages? (2) Does developmental stage variation reflect phylogenetic structure? (3) Is there an association between developmental stage variation and different life forms (epiphytic versus terrestrial) or on bioclimatic origins (i.e., tropical versus temperate)? (4) Is there an association between developmental stage variation and pollination strategies (i.e., rewarding versus deceptive)?

For these aims, we investigated and categorized stages of ovule development at anthesis for a representative sampling of the orchid family. Our survey included tropical and temperate members of all orchid subfamilies and species with contrasting pollination strategies and life forms.

MATERIALS AND METHODS

Sampling

In this study, we analyzed ovule developmental stages at anthesis in 94 species spanning the five orchid subfamilies (Apostasioideae, Cypridioideae, Vanilloideae, Orchidoideae, Epidendroideae; see Appendix S1). For 39 species, our observations were the first of these tissues, and we collected literature information for 51 species. From the literature survey, we only selected studies that included detailed images. To the data set, we also added unpublished data on ovule developmental stage of four species collected using scanning electron micrographs from the archive of one of the authors (M. R. Barone Lumaga). We also included species with different life forms (terrestrial versus epiphytic), bioclimatic origins (tropical versus temperate), and pollination strategies (rewarding versus deceptive). We assigned species to temperate and tropical categories if the species had all, or most, of their range falling in a temperate or tropical area, respectively. By tropical areas, we mean nonseasonal tropical environments, that represent the habitat where the majority of orchid species are found. Species that are widespread through temperate and tropical climes were assigned to a mixed state and were excluded from comparisons.

Light microscopy observations and ovule developmental stages categorization

Anthetic ovaries from unpollinated flowers were collected in Lillie's buffered neutral formalin (Lillie, 1965), dehydrated in a graded ethanol series, and embedded in Leica Historesin (Heraeus Kulzer, Hanau, Germany). Cross sections of the middle region of the ovary (5 μm thick) were cut on a rotary microtome (Leica). Serial sections were stained with toluidine blue O (Sakai, 1973) and mounted in Entellan synthetic resin (Merck Millipore, Burlington, MA, USA). Photomicrographs were taken with an Olympus BX 51 photomicroscope equipped with an Olympus DP71 camera.

Our data set merged observations from scanning electron microscopy (SEM) and light microscopy, which may potentially create a problem because these two techniques allow the observation of different portions of the ovules: the

external surface and, if present, the integuments (SEM) or the external and internal ovule sections (light microscopy). However, it has been widely demonstrated that stages of integument development have a strict correspondence with the developmental stage of the germline tissue (Tsai et al., 2008; Barone Lumaga et al., 2019), hence allowing the categorization of four stages of ovule development identifiable through either of the two techniques: stage 1, absence of ovules and presence of placental protuberances; stage 2, ovule primordia with the subdermal terminal cell differentiating into an archesporial cell bordered by nucellar epidermis and initials of the inner integument sometimes visible; stage 3, ovule primordia with mother cell of the megaspore and cell divisions of the dermal layer leading to the formation of the inner and outer integuments; stage 4, mature ovules (fully developed embryo sac and integuments) (see Figure 2).

Phylogenetic analysis

To build a phylogenetic tree as background for a phylogenetic generalized least square (PGLS) analysis, we harvested sequences for three plastid markers (*matK*, *rbcL*, and *psaB*) and one nuclear marker (ribosomal ITS) from GenBank for 83 of the studied species, choosing sequences from the same species or the same genus when not available. We then aligned each marker using MAFFT (Katoh et al., 2019) employing the strategy Auto and the option Leave gappy regions. Alignments were then inspected and concatenated using Mesquite (Maddison and Maddison, 2006). To build a dated phylogeny from our sequences, we employed BEAST v. 2.6 (Bouckaert et al., 2019). *Neuwedia veratrifolia* was selected as an outgroup to force an ingroup monophyly constraint. We then calibrated the root node of the Orchidaceae using secondary calibrations from Chomicki

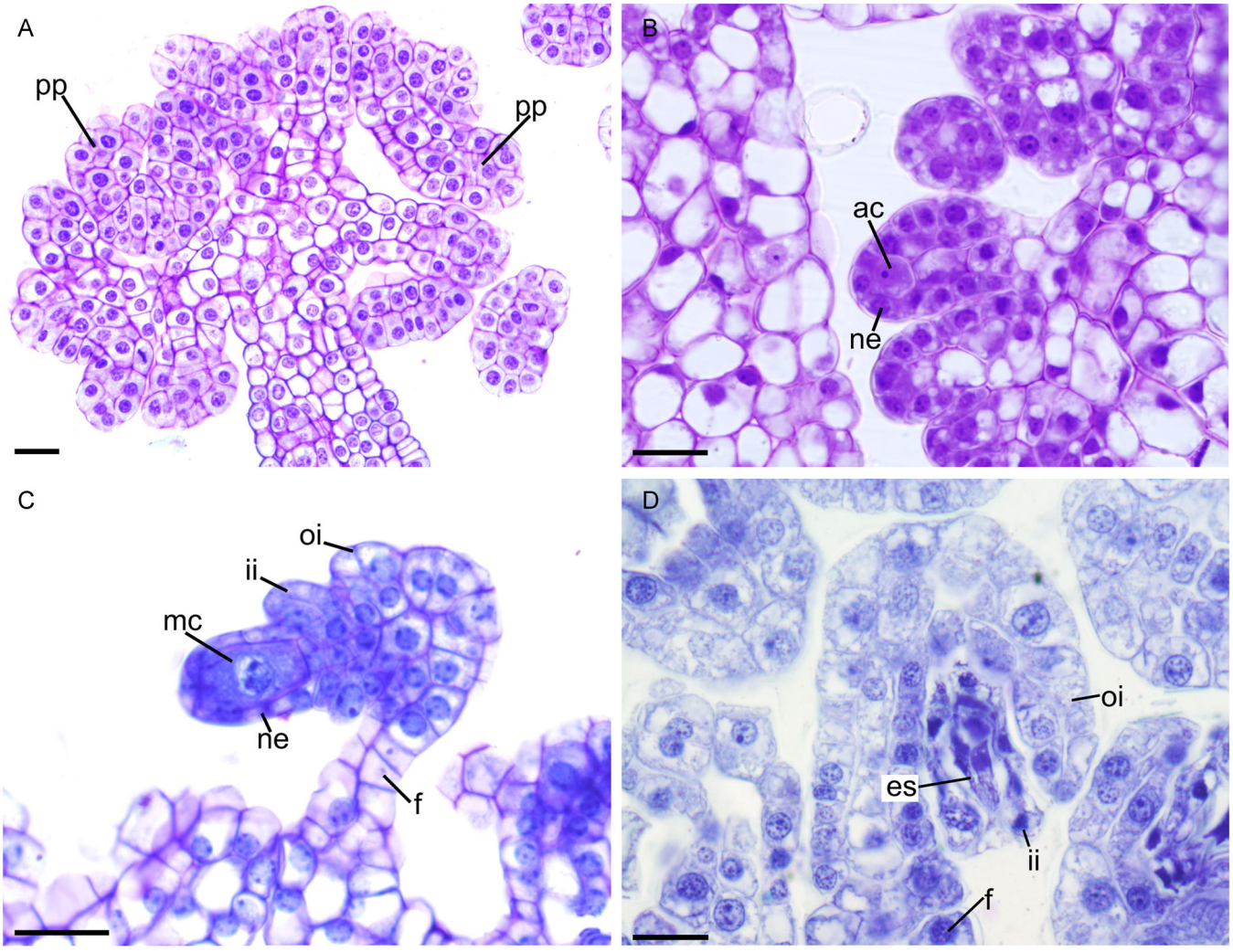

FIGURE 2 Light micrographs of the four stages of ovule development at anthesis. (A) Stage 1. Absence of ovules and presence of placental protuberances (pp) in *Arundina graminifolia*. (B) Stage 2. Ovule primordia with the subdermal terminal cell differentiating into an archesporial cell (ac) with an evident nucleus bordered by the nucellar epidermis (ne) in *Epidendrum fulgens*. (C) Stage 3. The archesporial cell does not divide and directly becomes the mother cell of the megaspore (mc), and cell divisions of the dermal layer lead to the formation of the inner (ii) and outer integuments (oi) of the ovule in *Ludisia discolor*. (D) Stage 4. Mature ovule with embryo sac (es) in *Prescottia oligantha*. f = funiculus. Scale bars = 20 μm

et al. (2015), setting the prior as a normal distribution with offset of 93.7 and SD of 6. We also calibrated the higher Epidendroideae node (excluding Neottieae and Sobraliinae) using the fossils *Dendrobium winkaphyllum* and *Earina fouldenensis* (Conran et al., 2009) by setting the prior as a gamma distribution with offset of 20 and beta of 4.5. Four unlinked site models (GTR plus gamma) were used for the four gene partitions (*matK*, *rbcL*, *psaB*, and ITS), while two separated uncorrelated lognormal clock models were used for the plastid and nuclear partition. The Markov chain Monte Carlo (MCMC) was run for 10,000,000 generations sampling every 1000th generation. Convergence was assessed using Tracer v 1.5.0 (Rambaut et al., 2018), with an effective sample size for the parameters higher than 200. Tree samples from three independent runs were combined using LogCombiner v1.10, removing the first 10% of the trees as burn-in. A maximum clade credibility tree was obtained using TreeAnnotator. The maximum clade credibility tree and 100 random trees from the posterior are available in FigShare (https://doi.org/10.6084/m9.figshare.9700250).

PGLS and ancestral state reconstruction

We used our phylogeny to reconstruct the evolutionary history and the phylogenetic signal of the ovule development stage trait using two scoring approaches. In the first approach, we scored the ovule development stage trait as a continuous trait. Species with ambiguous states were scored as having an intermediate state (i.e., 2/3 was scored as 2.5). In the second approach, we scored the trait as categorical, with species with ambiguous states with equal probability (0.5 and 0.5). Ancestral state reconstruction for the continuous trait was conducted using a maximum likelihood approach implemented using the function fastAnc from the package phytools (Revell, 2012). For the categorical trait, reconstruction was conducted using stochastic mapping and the function make.simmap from the package phytools. The mapping was repeated 100 times and run using an all-rates-different model, which allows transitions between all possible states, as well as an ordinated model, which only allows transitions between adjacent states, both with empirically estimated stationary frequencies.

Phylogenetic signal was inferred for the continuous scoring of the ovule development stage trait using the function phylosig from the package phytools. We inferred both Blomberg's K and Pagel's λ, and we used both the maximum clade credibility (MCC) tree and the full set of trees from the posterior of the MCMC.

To distinguish the contribution of ecological factors (i.e., life form, bioclimatic origin, and pollination strategy) from the effect of phylogenetic relationships, a PGLS analysis was conducted using R. We used the continuous coding of the ovule development stage trait, since correlations between categorical traits are problematic (Maddison and Fitzjohn, 2015). The function pgls from the package caper (Orme et al., 2012) was used to test three models with the ovule developmental stage (treated as a continuous variable) predicted by pollination strategy (rewarding versus deceptive), bioclimatic origin (temperate versus tropical versus mixed) and life form (terrestrial versus epiphytic). A model including the interaction between pollination strategy and life form was also tested to test the potential for trade-offs. The lambda value that adjusts the expected covariance due to shared evolutionary history was optimized using maximum likelihood for each model. The interaction analyses were run not only on the MCC tree, but also on 100 random trees from the post burn-in MCMC sample to account for uncertainty in dating and topology, as well as to control for the few poorly supported nodes in our MCC tree topology.

Because the absence of significant differences in PGLS can eventually be the consequence of an overall correlation between ecological and phylogenetic signals, we also compared ovule developmental stages at anthesis (treated as a categorical variable) in species with different life forms (terrestrial versus epiphytic), bioclimatic origins (tropical versus temperate) and pollination strategies (rewarding versus deceptive) using a χ^2 test. For this analysis, we used the whole data set and, when possible (i.e., when a sufficient number of species was available), within subfamilies.

Finally, to test for the effect of sampling on the results, we ran the interaction model between pollination strategy and life form using randomly reduced sampling with 80% of the taxa and 60% of the taxa. This random sampling was repeated 100 times.

RESULTS

We found that ovule developmental stage at anthesis is variable among the 94 investigated orchid species, ranging from an almost complete absence to the presence of mature ovules (see Appendix S1 and Figure 3). Light micrographs of the 39 species that we observed are in Appendices S2 and S3. Phylogenetic analysis results in a well-resolved tree, with only 10 nodes receiving support lower than 0.7 posterior probability (PP). Most of the subtribes are monophyletic. Vanilloideae are retrieved as sister to Cypripedioideae plus Epidendroideae and Orchidoideae. *Cleistes* (Pogonieae) is sister to *Vanilla* (Vanilleae) (0.98 PP). *Cypripedium* is sister to *Phragmipedium* plus *Paphiopedilum* (0.98 PP). The mycoheterotrophic *Epipogium aphyllum* is oddly retrieved as sister to Orchidoideae, instead of within Epidendroideae (0.98 PP), likely because of several plastid DNA rearrangements following the loss of photosynthetic function. Within Orchidoideae, Cranichideae are sister to Orchideae. The relationships within the Epidendroideae are less resolved. Neottieae are sister to Sobralieae plus a clade of Arethuseae, Cymbidieae, Malaxideae, Epidendreae and Vandeae. Some unorthodox relationships are found, such as a clade of Malaxideae and Vandeae (0.64 PP) sister to Epidendreae (0.78 PP), which is in turn sister to Cymbidieae (0.45 PP). However, these relationships are only poorly to

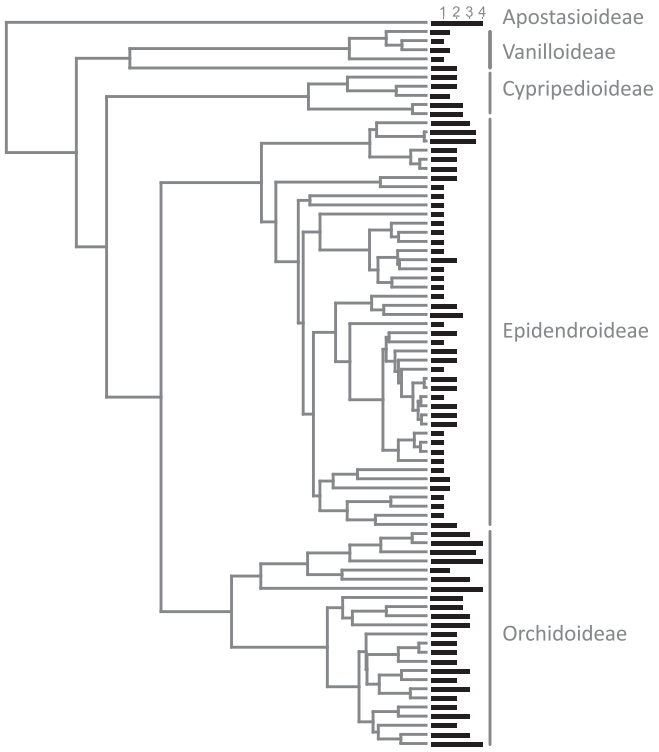

FIGURE 3 Variation in ovule developmental stage across phylogeny with bars indicating the highest ovule developmental stage for each species. Lowest, stage 1; highest, stage 4

moderately supported in our analyses and were not retrieved in other phylogenomic analyses of the Orchidaceae (Givnish et al., 2015; Kim et al., 2020).

The 95% highest posterior density (HPD) for the age of the crown group Orchidaceae spans from 102 to 79 million years ago (Ma) (Late Cretaceous). Crown group Vanilloidae are 84 to 45 Myr old, Cypripedioideae are 38 to 15 Myr old, Epidendroideae are 47 to 26 Myr old, and Orchidoideae are 68 to 38 Myr old (Appendix S4).

The ancestral state of the ovule developmental stage in the Orchidaceae is reconstructed in a similar way by both approaches (continuous and categorical) and all models, with only minor inconsistencies. The most recent common ancestor (MRCA) of the Orchidaceae is inferred to have had most likely a stage 2 ovule (2.5 in the continuous trait analysis). However, this result has high uncertainty in all methods. Transition to stage 1 is inferred to have happened at the crown node of Epidendroideae as well as at the crown node of the genus *Vanilla*, while transition to stage 3 is inferred to have happened at the crown node of Orchidoideae (Figure 4).

The phylogenetic signal analysis shows a very high Pagel's λ of 0.92 for the MCC tree, and between 0.81 and 1 for the posterior sample, while Blomberg's K is 0.53 for the MCC and between 0 and 0.8 for the posterior sample (see Appendix S5 for histograms of the distributions).

Rewarding orchids have more developed ovules at anthesis than deceptive orchids (Figure 5A). This difference was statistically significant when analyzing the whole data set ($N = 89$; Pearson $\chi^2 = 22.379$; df = 6; $P = 0.001$), the reduced data sets only including Orchidoideae ($N = 25$; Pearson $\chi^2 = 12.591$; df = 5; $P < 0.028$) and Epidendroideae ($N = 51$; Pearson $\chi^2 = 18.521$; df = 5; $P = 0.002$). The PGLS analysis confirmed that rewarding and deceptive species have significantly different ovule developmental stages ($P = 0.00549$).

Terrestrial orchids have more developed ovules at anthesis than epiphytic orchids (Figure 5B). This difference is significant by analyzing the whole data set (N = 91; Pearson $\chi^2 = 30.143$; df = 6; $P < 0.001$) and a reduced data set only including Epidendroideae ($N = 53$; Pearson $\chi^2 = 14.254$; df = 5; $P = 0.014$). The PGLS analysis showed no significant difference between life forms ($P = 0.108$).

Temperate orchids have more developed ovules at anthesis than tropical orchids (Figure 5C). This difference was significant when analyzing the whole data set ($N = 90$; Pearson $\chi^2 = 44.110$; df = 6; $P < 0.001$) and a reduced data set only including Epidendroideae ($N = 55$; Pearson $\chi^2 = 31.980$; df = 5; $P < 0.001$). These two comparisons were not performed in Orchidoideae due to the absence of epiphytic species and the limited number of available tropical species in our data set. The PGLS analysis showed no significant difference between bioclimatic origins ($P = 0.669$).

A model with an interaction between pollination strategy and life forms showed a significant interaction, with terrestrial orchids having larger differences between rewarding and deceptive species than epiphytic orchids

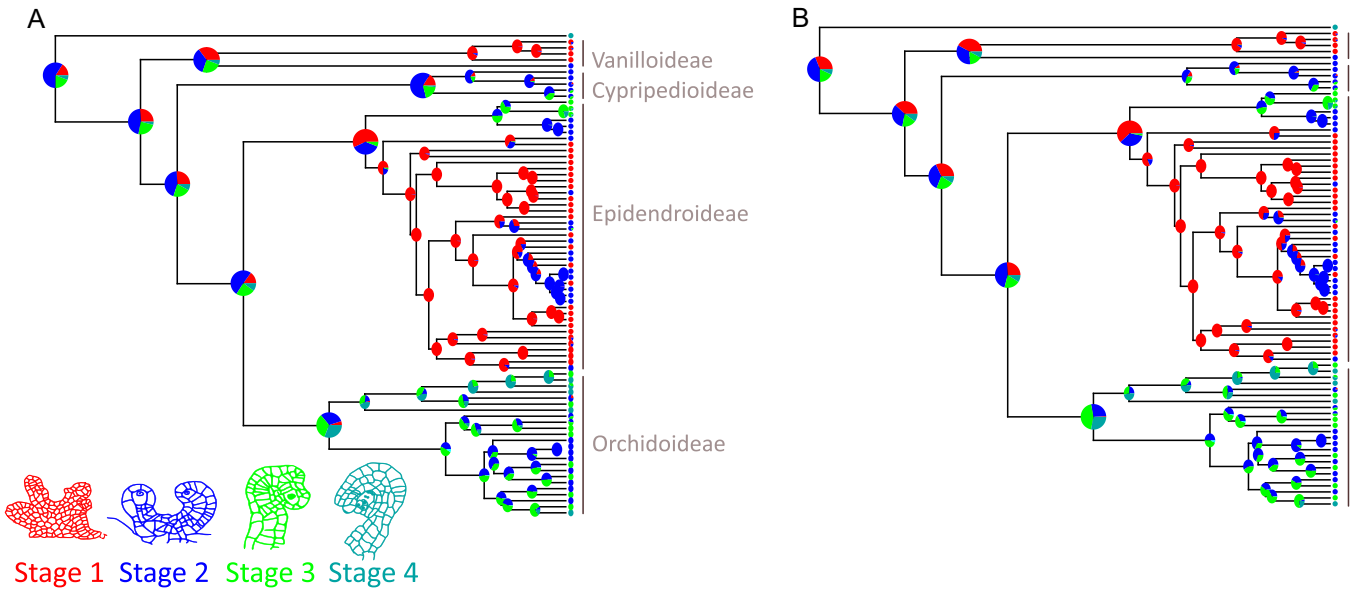

FIGURE 4 Reconstruction of ancestral state of ovule development stage in the Orchidaceae. (A) Ovule development stage trait scored as a continuous trait (species with ambiguous states scored as having an intermediate state, i.e., 2/3 is scored as 2.5). (B) Ovule development stage trait scored as categorical (species with ambiguous states scored with equal probability: 0.5 and 0.5)

(interaction $P = 0.0012$; Appendix S6). The significance of the interaction term was robust to topology and dating uncertainties (Appendix S7) and was retrieved in 93% of the analyses with 80% of randomly chosen samples and in 79% of the analyses with 60% of randomly chosen samples (Appendix S8).

DISCUSSION

The evolutionary and ecological bases underlying the common occurrence of flowers with under-developed ovules in orchids have been largely overlooked. This lack is surprising considering that ovules represent one of the main reproductive investments of flowering plants (Delph, 1999; Obeso, 2002; Strelin and Aizen, 2018) and that orchids are one of the most species-rich plant families. In this study, we started to fill the gap by analyzing ovule developmental stages at anthesis in 94 orchid species (with new data for 43 species). With this data set, we found that ovule developmental stages at anthesis are highly variable, ranging from placental protuberances to mature mega-gametophytes, and can be shaped by ecological factors such as life form, bioclimatic origin, and pollination strategy. Large biodiverse families such as Orchidaceae may impose several constraints on studies aiming to infer broad patterns, and the number of species included in this data set clearly represents a small proportion of the estimated 28,000 species of Orchidaceae (Christenhusz and Byng, 2016). Within this limitation, however, our study included species representing main orchid clades (17 of the 23 recognized tribes; Chase et al., 2015), occurring in different regions, with diverse ecological interactions and allows us to account for a broad range of ecological and reproductive strategies. We further confirmed the representativeness of our sample set and robustness of results by using randomly reduced sampling (80% and 60% of accessions). Still, we recognize that large knowledge gaps for several orchid lineages (e.g., *Cymbidium*, *Bulbophyllum*, *Dendrobium*) need to be filled in future research.

To what extent does ovule development vary across orchid lineages?

Some variability in ovule developmental stage at anthesis was already reported (e.g., Arditti, 1992). The picture that arises from our data set clearly confirms that ovule development at anthesis is highly variable in orchids. Notably, this variation also occurs within tribes (e.g., from stages 1 to 4 in Neottieae, from stages 1 to 2 in Epidendreae, and from stages 2 to 4 in Orchideae) and among related species, suggesting that the stage of ovule development at anthesis can be readily modified in response to natural selection (Appendix S1 and Figure 3).

Does the variation in ovule developmental stages reflect phylogenetic structure?

To achieve clear evidence of the status of ovule development in the Apostasioideae is difficult because of the reduced number of species and their rarity. According to the literature (Kocyan and Endress, 2001) on two species of *Neuwiedia*, one of the two genera of the Apostasioideae, ovule development is almost complete at anthesis. This

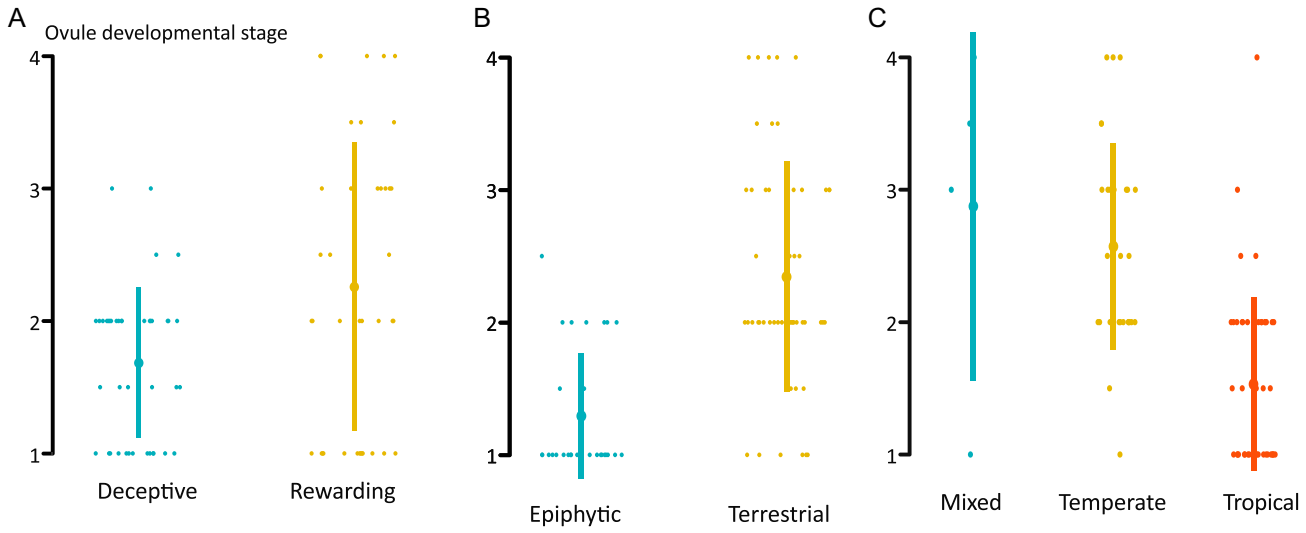

FIGURE 5 Variation in ovule developmental stage among species with (A) different pollination strategies (deceptive versus rewarding), (B) life forms (epiphytic versus terrestrial), and (C) bioclimatic origin (temperate versus terrestrial) with actual data points (small circles) and the estimate of the mean (dot on colored vertical bar) and 95% confidence interval (colored vertical bar)

finding suggests that members of Apostasioideae have the same ovule developmental stage at anthesis as some members of Asparagales such as Iridaceae and Hypoxidaceae (i.e., Vos, 1948; Rudall, 1994). However, already in the early-divergent subfamily Vanilloideae, ovules at anthesis are clearly under-differentiated, being at stage 1 (absence of ovules and presence of placental protuberances) or stage 2 (ovule primordia with the subdermal terminal cell differentiating into an archesporial cell). In the clade including Cypripedioideae, Epidendroideae, and Orchidoideae, ovule development is highly variable (from stage 1 to 4) among lineages (Figure 3).

Our ancestral state reconstruction analyses suggests that the ability to keep ovules under-differentiated at anthesis arose early in orchids' evolutionary history (at least between 84 and 45 Ma; see Figure 4 and Appendix S4) and that undifferentiated ovules at anthesis might be a synapomorphy of Orchidaceae as a whole or of non-Apostasioid orchids. Since Orchidaceae are sister to the rest of the Asparagales, selecting an outgroup for our analysis is limited by the sparse knowledge of ovule development in the order (data available for only a few families/species). Still, by generalizing the few available records of fully developed ovules in Asparagales (Vos, 1948; Rudall, 1994), the more likely scenario is that under-differentiated ovules evolved in orchids only after the basal divergence of the Apostasioideae. Further under-differentiation evolved independently in *Vanilla* and at the base of the Epidendroideae. Our phylogenetic signal analyses indicate that ovule development has a strong phylogenetic structure, though the sparse sampling in our phylogeny might have inflated the values of Pagel's λ. At the same time, transitions between stages of ovule development among lineages seem to have occurred frequently and within a narrow temporal frame (<5 Ma) (Appendix S4), and the low values of Blomberg's K (<1) we retrieve suggest that most of the variance is still partitioned within clades.

Is there an association between developmental stage variation and different life forms (epiphytic versus terrestrial) or on bioclimatic origins (i.e., tropical versus temperate)?

The orchid family colonized a great diversity of biomes giving rise to tropical (epiphytic and terrestrial) and temperate (almost exclusively terrestrial) clades. A recent survey shows that life form is a driver for the evolution of aerodynamic traits of seeds in orchids because terrestrial species that release seeds closer to the ground have more aerodynamic seeds, likely due to a stronger selective pressure to increase seed-dispersal efficiency (Fan et al., 2019). To understand whether life form or bioclimatic origin can be drivers for the anthetic ovule developmental stage, in our study, we firstly used the complete data set including 94 species to compare epiphytic versus terrestrial orchids and tropical versus temperate orchids. We found that epiphytic orchids have less developed ovules than terrestrial orchids and that tropical orchids have less developed ovules than temperate orchids (Figure 5B, C). However, these two comparisons are correlated because all epiphytic orchids in our data set (and by far the majority of all existing epiphytic orchids) inhabit tropical regions. Indeed, although this correlation does not allow us to disentangle the effects of life form and bioclimatic origin, the observed differences in anthetic ovule developmental stage between tropical and temperate orchids fit a hypothesis previously stated by Swamy (1943) and more recently tested by Barone Lumaga et al. (2019). The idea is that tropical orchids experiencing a long growing season can afford the extended

time necessary for complete postpollination ovule development, whereas temperate species, with a short growing season must achieve partial megagametophyte development before anthesis to complete the reproductive cycle within the season (Swamy, 1943; Barone Lumaga et al., 2019).

When taking into account the phylogenetic signal, i.e., in the PGLS analysis, the detected differences between orchids with different life forms and different bioclimatic origins become nonsignificant. This result is likely due to strong niche conservatism in the orchids; that is, 15 of the 17 investigated tribes only encompass species with the same life form and/or bioclimatic origin, hence reducing the number of phylogenetically independent comparisons. However, the absence of significant differences in PGLS is not an indication that life form and bioclimatic origin cannot be ecological drivers for the stage of anthetic ovule development. Rather, and most likely, the absence of significant differences can be the consequence of an overall correlation between the ecological and the phylogenetic signal, an issue that is unlikely to be resolved even by increasing the sample size.

Is there an association between developmental stage variation and pollination strategies (i.e., rewarding versus deceptive)?

It has been hypothesized that, in orchids, pollination-triggered ovule development ensures efficient investment in megagametophyte and ovary maturation for fertilization because of the low probability of pollination by highly specified pollinators (O'Neill, 1997). However, comparative studies have shown that specialized and generalist orchid species do not differ in terms of fruiting failure, while significant differences were found between rewarding and deceptive orchids (Tremblay et al., 2005; Scopece et al., 2009). Given that rewarding orchids are less exposed to the risk of fruiting failure than deceptive ones, they should have a smaller advantage in keeping their ovules under-developed. Accordingly, we found that in our statistical comparison rewarding species have more developed ovules at anthesis than deceptive species (Figure 5A). This finding is independent from the phylogenetic signal as confirmed by the PGLS analysis, likely due to the fact that in the investigated data set, transitions between deceptive and rewarding pollination strategies occurred independently in seven tribes. The observed differences are consistent in the two larger orchid subfamilies (Orchidoideae and Epidendroideae) and are larger in temperate (terrestrial) than in tropical (epiphytic) species (see Figure 3). Indeed, tropical and epiphytic species have less variance in the anthetic ovule developmental stage (most of the species ranging between stages 1 and 2) than temperate and terrestrial species (ranging from stage 1 to 4).

The correspondence between ovule developmental stage and pollination strategy is very interesting and can be considered a facilitating factor for the evolution of the extraordinarily high number of orchid species will deceptive pollination strategies. Keeping ovules under-differentiated greatly decreases the waste of resources due to fruiting failure, thus reducing the investment loss in species with a low fruit set such as the deceptive orchids. The early evolution of under-differentiated ovules may thus represent a preadaptation that can explain the unique abundance of deceptive flowers in orchids. Since transitions between pollination strategies are often observed between species that have recently diverged (Johnson et al., 2013; Cardoso-Gustavson et al., 2018), we may also expect transitions in ovule development stages occurring at these short evolutionary scales, potentially playing a role in building reproductive isolation mechanisms based on pollen–ovule interaction.

CONCLUSIONS AND PERSPECTIVES

Despite a broad phylogenetic signal, variation in ovule development was observed among and within orchid clades. Interestingly, this variation is clearly linked to ecological factors: ovules are less developed in tropical than in temperate species, and in deceptive rather than in rewarding orchids. These results strongly suggest that, in orchids, the ovule developmental stage at anthesis can be shaped by ecological factors and can thus be selected to optimize female reproductive investment.

Orchids offer many opportunities for studying the genetic and physiological processes of ovule development. They include a plethora of species that show natural deviation in ovule developmental stages at anthesis and can provide insights on the pathway controlling germline progression and the discovery of molecules involved in the crosstalk between the two (sporophytic and gametophytic) generations. In this context, orchid species arrested at different stages of ovule development at anthesis represent the natural counterpart of the mutants from model species (as *Arabidopsis*) that have been successfully employed to identify master genes of ovule development (e.g., Elliott et al., 1996). Several genes, that in *Arabidopsis* have been demonstrated to be key regulators for acquiring germline identity and for entering meiosis and committing to germline fate (Pinto et al., 2019), can now be searched for homologous counterparts in the recently released orchid genomes (Cai et al., 2015; Zhang et al., 2017). Studies on their regulation can clarify the processes responsible for explaining why germline cells enter meiosis in some orchid species and not in others and how this process can be differently selected in response to ecological pressures.

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AUTHOR CONTRIBUTIONS

J.L.S.M., S.C., and G.S.: conceptualization; JLSM, FP and MRBL; M.C. and G.S.: formal analysis; G.S. and S.C.: supervision; J.L.S.M., M.R.B.L., F.P., and M.C.: visualization; all authors: writing – original draft; all authors: writing – review and editing.

DATA AVAILABILITY STATEMENT

The maximum clade credibility tree and 100 random trees from the posterior are avaiable in FigShare (https://doi.org/10.6084/m9.figshare.9700250).

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

Appendix S1. Species included in the study with references for image information, life form, bioclimatic origin, pollination strategy, and stage of ovule development.

Appendix S2. Light micrographs of anthetic ovules of orchid species from the subfamilies Vanilloideae, Cypripedioideae, and Orchidoideae.

Appendix S3. Light micrographs of anthetic ovules of orchid species from the subfamily Epidendroideae.

Appendix S4. Maximum clade credibility tree for Orchidaceae, based on plastid markers *matK*, *rbcL*, and *psaB* and nuclear marker ribosomal ITS for 83 orchid species.

Appendix S5. Histograms of the distributions for the maximum clade credibility (MCC) of the posterior sample.

Appendix S6. Variation in ovule developmental stage between ecological groupings with actual data points (small circles and triangles) and the estimate of the mean (dot on colored vertical bar) and 95% confidence interval (colored vertical bar).

Appendix S7. Results of the interaction model of the phylogenetic generalized least square (PGLS) on the 100 random trees from the posterior sample.

Appendix S8. Results of the resampling analysis with the phylogenetic generalized least square (PGLS) model with pollination mode, habit, and their interaction. Sampling proportion used (80% or 60%) is indicated.

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